TARGETING THE GENETIC AND MOLECULAR ROLE OF ABCG2 FOLLOWING TRAUMATIC BRAIN INJURY

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Traumatic brain injury (TBI) is a leading cause of death and disability. Despite its overwhelming burden on sufferers, their families, and the health-care system; there are no pharmacologic treatments that improve long term clinical outcomes. Drug development for TBI has focused on mitigation of factors that contribute to the persistent secondary injury from TBI. These target reactive oxygen species generation, cerebral edema, excitotoxicity, among others. Genomics has been a productive area to support TBI drug development, and pharmacogenomics (PGx) - based drug use and development has gained utility in critical care and TBI. This helps to identify new targets for treatment and/or prognostication, and to provide targeted therapy based on an individual's genomics. Our objective was to evaluate the role of drug transporters, particularly the ATP-Binding Cassette transporter, ABCG2, following severe TBI. We measured expression of transporters in experimental TBI, evaluated the association of a missense genetic variation in *ABCG2* with clinical outcomes from TBI, and studied the change in disposition of the ABCG2 substrate, uric acid (UA), in patients with TBI. We found that overall transporter expression tended to decreased acutely following TBI, exemplified by ABCG2 expression, which was significantly decreased early follow-

ing injury with a rapid return to baseline. The missense variation in the *ABCG2* gene, c.421C>A, was found to be associated with improved outcomes following severe TBI in younger patients, suggesting that ABCG2 dysfunction is neuro-protective. Finally, we discovered that patients with severe TBI who carry a variant allele at *ABCG2* c.421C>A have measurable decrease in UA transport in the CNS. These findings suggest that UA is protective following TBI, which may be related to its role as an antioxidant. Future study in this area can include further development of animal models for TBI that can mimic human levels of UA, which are about 10-fold higher in humans than in most other mammals. They may also include prospective observational trials to validate the findings of UA's association with ABCG2 and outcomes. Clinical trials of therapeutic UA or the UA precursor, inosine, may also be warranted in patients suffering from severe TBI.

Keywords: ABCG2, TBI, Transporters, Pharmacogenomics, Neurology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

This dissertation is dedicated to my children: Maxwell, Eleanor, and Penny; you remind me that there is always reason to keep going.

Five years ago I came to Pittsburgh to pursue scientific training and a PhD. Reflection on that time is overwhelming; I have been very fortunate to have had so many positive experiences. I owe incredible gratitude to my advisor and mentor, Dr. Philip Empey, who has given me countless opportunities through my time as a PhD student. Experiences working with Dr. Empey have shaped my scientific background to what it is today, and he will always represent the level of excellence and integrity that I want to achieve as a scientist. I owe significant gatitude to my friends and colleagues from the Empey lab: Fanuel, Karryn, Kacey, Ashley, and Molly; all have heped shape my scientific training and my dissertation work. I would be remiss if I did not acknowledge the tremendous support from faculty and staff at the University of Pittsburgh School of Pharmacy and my dissertation committee: Drs. Ma, Kochanek, Poloyac, and Conley. Finally, I am incredibly humbled by the hundreds of research participants whose time and sacrifice made my research possible.

Lastly, this work has been made possible second to the love and support from my parents Brenda, Marcus, and Alan that encouraged my curiosity and desire to pursue

higher education. But most of all, I owe this accomplishment to the dedication and sacrifice of my wife, Missy.

ABBREVIATIONS

ABC:	ATP-Binding Cassette
ADR:	Adverse Drug Reaction
BBB:	Blood-Brain Barrier
CCI:	Controlled Cortical Impact
CNS:	Central Nervous System
CPP:	Cerebral Perfusion Pressure
CSF:	Cerebro-Spinal Fluid
CT:	Computed Tomography
DAI:	Diffuse Axonal Injury
EDH:	Epidural Hematoma
FDR:	False Discovery Rate
GCS:	Glasgow Coma Scale
GOS:	Glasgow Outcome Scale
ICP:	Intracranial Pressure
ICU:	Intensive Care Unit
IPH:	Intraparenchymal Hemorrhage
IVH:	Intraventricular Hemorrhage
MAF:	Minor Allele Frequency
MRI:	Magnetic Resonance Imaging

mRNA:	Messenger RNA
MVA:	Motor Vehicle Accident
OBTT:	Operation Brain Trauma Therapy
PGx:	Pharmacogenomics
PND:	Post-Natal Day
PTD:	Post-Traumatic Depression
PTE:	Post-Traumatic Epilepsy
PTS:	Post-Traumatic Seizures
ROS:	Reactive Oxygen Species
SAH:	Subarachnoid Hemorrhage
SDH:	Subdural Hematoma
SLC:	Solute Carrier
SSRI:	Selective Serotonin Reuptake Inhibitor
TCA:	Tricyclic Antidepressant
TBI:	Traumatic Brain Injury
UA:	Uric Acid

1.0 INTRODUCTION AND BACKGROUND

[Adams SM, et al. *Pharmacogenomics* 2017]

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See Appendix 3 Figure C1

1.1 TRAUMATIC BRAIN INJURY OVERVIEW

Traumatic brain injury (TBI) is a leading cause of morbidity and mortality among children and young adults, contributing to nearly a third of all injury-related deaths.[2] Lending to the complexity of TBI is the dearth of treatments that improve clinical outcomes.[3] This problem requires the study of topics ranging from trauma, resuscitation, neuroscience, and molecular biology to understand the disease processes that drive the neurologic damage that often leads to death or long-term disability in those who suffer from TBI.

Our focus is on the development of novel treatment and prognostication modalities for TBI by studying how changes in genetics impact TBI pathophysiology and outcomes. Drug development can involve drug re-purposing and new drug development, often drawing on pharmacotherapy from other disease states and patient-specific factors. This use of precision medicine in critical care is likely to support enhanced drug development efforts in TBI.[4] We provide a review of the field of TBI pharmacotherapy and current status of precision medicine as it applies to pharmacotherapy of TBI. We also discuss the pathophysiology of TBI in addition to the standard and innovative approaches to pharmacotherapy.

1.1.1 Epidemiology

Traumatic brain injury contributes to nearly 30% of all injury-related deaths.[5] The most common causes for non-fatal TBI are from falls, motor-vehicle accidents (MVA), and blunt injuries or blows to the head, which account for nearly 70% of all TBIs.[2, 5, 6] Leading causes of fatal TBI vary with age group, with falls predominantly affecting individuals 65 years and older, self harm for individuals 25-65, MVA for 5-24, and

assault for children up to 4 years old.[5] Non-fatal TBI follows similar patterns, with falls driving ED vists for everyone except for those age 15-24, for whom being struck with an object is the leading cause.[5] TBI-related hospitalizations are driven by falls in children up to 14 years old and in adults over 45, while other age groups are primarily hospitalized for MVA.[5] Collectively, these injuries account for nearly three million emergency department visits, hospitalizations, and deaths every year in the United States.[5, 6] It is critical that drug development for TBI account for different ages as the causes of injury and expected outcomes vary with age.[7]

1.1.2 Measurements of Injury Severity

Traumatic brain injury describes a diverse group of injuries ranging from concussions (mild TBI) to moderate and severe injuries (e.g. MVA, combat-related injuries, severe sports injuries, etc.). There are multiple competing clinical definitions of how the breakpoints of TBI severity should be defined.[8] Commonly used TBI severity measurements include the Glasgow Coma Scale (GCS) score, the Barell Type, the Injury Severity Scale score, and the Abbreviated Injury Scale score, among others.[8, 9] The GCS is commonly employed due to its simplicity, ability to be measured very early following injury, and widespread familiarity. The GCS score is also validated for use in clinical populations and is a strong predictor of mortality.[10] The complete GCS score contains measures of motor response, eye opening, and the ability to communicate verbally or make vocalizations (**Table 1.1**).[11]

Table 1.1: The Glasgow Coma Scale[11] score is a composite measurement ranging from 3-15. It comprises the sum of observed patient eye opening, response to verbal commands, and motor response to stimuli.

Measurement	Finding	Score
Eye Opening Response	Spontaneous opening with blinking at baseline	4
	Opens to verbal command speech or shout	3
	Opens to pain not applied to face	2
	None	1
Verbal Response	Oriented	5
	Confused conversation but able to answer questions	4
	Inappropriate responses; words discernible	3
	Incomprehensible speech	2
	None	1
Motor Response	Obeys commands for movement	6
	Purposeful movement to painful stimulus	5
	Withdraws from pain	4
	Abnormal (spastic); flexion decorticate posture	3
	Extensor (rigid); response decerebrate posture	2
	None	1

Ideally, GCS should be collected as soon as possible post injury by emergency medical services or in the emergency department with follow up collections throughout the patient's hospitalization.[12] A limiting factor and disadvantage of the GCS is that it should be collected in the absence of pharmacologic agents, especially sedating agents. Failure to obtain the uncontaminated GCS may lead to falsely low scores, which may prevent accurate injury classification.[13] The Injury Severity Scale, Barell Type, and Abbreviated Injury Scale may be collected later following injury, and may incorporate the impact of trauma to other body systems.[8] These do not limit data to brain injury, but can account for additional body regions, though severity information for the brain injury can be abstracted from the total score. Another robust

measurement for injury severity is derived from magnetic resonance imaging (MRI) or computed tomography (CT) imaging data.[14] This can allow the clinician to better understand the severity of the primary injury, the diffuse or local nature of the parenchymal damage, and the location and extent of hemorrhage.[14]

Correlations with outcomes are commonly found among each scoring method, though it is notable that GCS is most commonly used yet may not be the most correlated with outcomes.[9] This may be because it is often collected very early following injury at a time when the full breadth of the injury has not yet affected the patient, or it is collected when the patient is sedated.[15] Despite its drawbacks, GCS is commonly used in addition to imaging data to prognosticate in the clinic and to control for injury severity in TBI research. The GCS also has well established break-points for injury severity, where a score of 3-8 is classified as severe TBI, 9-12 as moderate TBI, and 13-15 as mild TBI.[8]

1.1.3 Measurements of Patient Outcomes

Outcomes from TBI are reported with the Glasgow Outcomes Scale (GOS) score and GOS-Extended (GOS-E), both developed between 1970 and 1980.[16, 17] These provide detail regarding patient status that can be assessed quickly and have historical use as outcome measures in clinical trials. Additional measures include the Disability Rating Scale score, Neuro-behavioral Rating Scale score, and numerous others.[18, 19, 20] These assess more granular measures of outcomes at the cost of more overhead required for collection.[18, 19, 20]

The GOS score is an ordinal scale ranging from 1-5 where 1=death, 2=persistent vegetative state, 3=severe disability, 4=minor disability, and 5=no disability.[16] In many clinical trials, the GOS is dichotomized with a sliding scale, most often defin-

ing a favorable outcome as GOS > 3, and an unfavorable outcome as GOS ≤ 3.[21] The dichotomous measurement provides practical interpretation of findings with some notable drawbacks, including loss of robustness and statistical power.[21] Lu and colleagues suggest that GOS evaluated as an ordinal variable without dichotomization may be less sensitive to data errors as well (e.g. a patient given a 3 when 4 is more appropriate).[21] This requires more robust statistical methods and adds challenge to result interpretation, but is a reasonable way to improve the analysis of TBI outcomes.[21]

1.1.4 Traumatic Brain Injury Disease Processes

1.1.4.1 Pathophysiology and Secondary Injury

Traumatic brain injury consists of several disease processes with temporal relationship to the primary insult. The primary TBI relates to the physical injury, which can be blunt or penetrating. This leads directly to damage from compressing, tearing, and/or shearing of the parenchyma.[22] Furthermore, these injuries can lead to focal damage within the brain, or they can cause varying degrees of diffuse injury. Diffuse axonal injury (DAI) is associated with widespread axonal injury due to internal stress from the primary injury, primarily resulting from shear stress from rotational acceleration.[23, 24] These are assessed clinically with head CT and MRI scans, though MRI is typically superior to CT scans in detecting DAI.[24] The primary injury requires immediate medical therapy to resolve external damage, remove debris, and attain hemodynamic stability. After resuscitation, attention must be diverted to preventing progressive neurologic damage that can drive poor clinical outcomes. This progressive response results from intrinsic factors related to injury repair, inflammation, and physiologic changes and is referred to as secondary injury.[25]

Hallmarks of secondary injury include changes in cerebral blood flow, loss of neurovascular auto-regulation, hypoxia, and metabolic dysfunction. Punctuating these pathways are progressive inflammation, damage from reactive oxygen species (ROS), and glutamate driven excitotoxicity.[25, 26] Despite their value in growing understanding of TBI, *in vivo* and *in vitro* TBI models have shown limited success in translating pharmacologic therapies into clinical practice.[27] Innovative approaches are critical to the successful discovery and development of TBI therapeutics. Methods that combine individual patient characteristics with "big-data" (e.g. genomic, metabolomic, transcriptomic) may provide the knowledge to eliminate the gap created by individual and injury heterogeneity.[28, 29, 30]

1.1.5 Current State of Drug Development for Severe Traumatic Brain Injury

In contrast to other disease states with heavy burden on those suffering and on society (e.g. cardiovascular disease), TBI does not have any available pharmacologic treatments that are known to improve clinical outcomes.[31] This has sparked substantial drug development effort, with a growing list of drugs entering pre-clinical and clinical trials. Efforts in TBI drug development have driven better understanding of the disease processes around TBI, but gaps in our knowledge of basic disease processes remain. This is driven by substantial injury heterogeneity and inter-individual variability in disease course, which challenges our full understanding of TBI.[31] The search for effective medications that improve severe TBI outcomes has included screening for novel molecules, and work in re-purposing existing agents for TBI.[31] This effort has involved translational research work involving pre-clinical *in vitro* and *in vivo* models, coupled with bio-banks and information repositories for patients suffering from TBI. One such proponent of this strategy of drug development for TBI is Operation

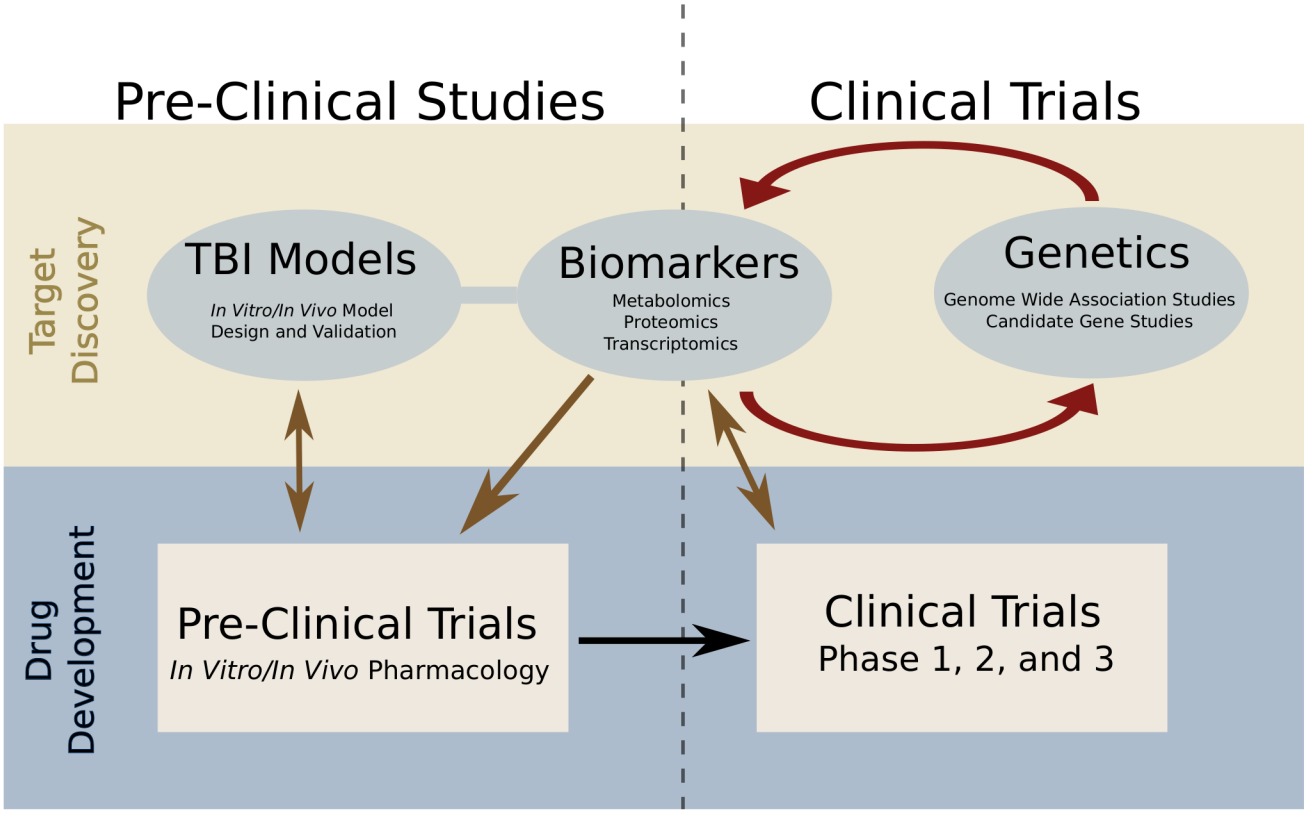

Figure 1.1: Drug development for TBI is a translational process. As depicted, a combination of translational studies that evaluate preclinical and clinical studies ranging from large-scale human genomic association studies with other clinical/preclinical techniques allows for rapid translation and validation of novel findings. These inform the progress of TBI pre-clinical models to hasten translation to human clinical trials.
Reprinted with permission from Pharmacogenomics[1] - See Appendix 3 Figure C1

Brain Trauma Therapy (OBTT), which incoroporates multiple animal TBI models spread across multiple research centers to study candidate drugs for TBI.[32] These efforts are summarized in **Figure 1.1**.

1.2 PRECISION PHARMACOTHERAPY FOR TRAUMATIC BRAIN INJURY

Traumatic brain injury pharmacotherapy focuses on 1) resuscitation, 2) management of the patient in the immediate post-injury phase, and 3) maintenance of the long-term complications from TBI.[12] Patients frequently require intubation and intensive monitoring, including neurologic assessments and frequent evaluation of cardio- and neuro-vascular parameters.[33] Medications used during patient hospitalization include sedatives, analgesics, and neuromuscular blocking agents.

1.2.1 Pharmacotherapeutics and Pharmacogenomics for TBI

As with any disease state for which pharmacotherapy is indicated, medications frequently do not work as expected. Adverse drug reactions (ADRs), toxicity, and inadequate response are common among medications, regardless of disease state.[34] Advances in genomics have opened the opportunity for the use of genomic data to tailor pharmacotherapy based on genetic variations (pharmacogenomics, PGx) in genes for drug metabolism, distribution, and drug action. PGx with regards to TBI promises the ability to tailor medication to patient baseline characteristics in addition to disease specific situations. The use of PGx in the ICU presents new challenges driven by the added complexity and heterogeneity of patient presentation and clinical course.[35, 36] Current PGx evidence supports its use to inform medication selection

and/or dose for seizure prophylaxis, analgesia, and sedation.[36, 37, 38]

1.2.1.1 Seizure Treatment and Prophylaxis

Post-traumatic seizures (PTS) can be clinical or sub-clinical in nature, and both types can contribute to secondary injury.[39] PTS occur within seven days in as many as 22% of patients with moderate and severe TBI.[12] Current treatment guidelines recommend the use of seizure prophylaxis in the first seven days post injury to prevent PTS. Based on work by Temkin and colleagues in 1990, the drug of choice for PTS prophylaxis has been phenytoin.[40] However, a growing body of literature supports levetiracetam as potentially equivalent or superior to phenytoin, [41] and potentially with neuro-protective benefits [42] - particularly when treatment is continued daily into the post-acute period.[43] PGx considerations for agents regarding PTS prophylaxis and treatment can be divided into genetic predictors of drug response (i.e. traditional PGx), and in PTS risk assessment (i.e. guided prophylaxis).

Pharmacogenomic predictors for phenytoin pharmacokinetics and response include the cytochrome P-450, family 2, sub-family C, member 9 (CYP2C9) and the major histocompatibility complex, class I, B (HLA-B), respectively. The Clinical Pharmacogenomics Implementation Consortium (CPIC) and Dutch Pharmacogenomics Working Group (DPWG) guidelines for phenytoin suggest that 1) patients with decreased CYP2C9 activity (e.g. CYP2C9 intermediate/poor metabolizers) receive a 25% to 50% lower maintenance dose, and 2) patients carrying the *HLA-B**15:02 haplotype should avoid phenytoin due to over four-fold increased risk for severe cutaneous adverse reactions.[37, 38] Siddiqui and colleagues also reported that *ABCB1* rs1045642 (GG) was associated with higher incidence of drug-resistance epilepsy (Odds Ratio: 2.66, 95%CI: 1.32, 5.38), which suggests that some anti-epileptic drugs' ability to cross the blood-

brain barrier (BBB) and/or the intestinal brush border may be impacted by ABCB1 function.[44] However, this finding has not been directly linked to phenytoin, and additional studies have not supported a significant contribution of variations in the *ABCB1* gene to phenytoin pharmacokinetics and pharmacodynamics.[45, 46] These PGx markers may be useful in TBI to identify patients who may require lower maintenance doses or an alternative agent, such as levetiracetam, during PTS prophylaxis and treatment.

The use of genetic markers to predict risk for PTS may help guide therapeutic decision making for individual patients. The adenosine A1 receptor (*ADORA1*) rs3766553 (AA) was associated with over five-fold increased risk for PTS in a study with 206 subjects with severe TBI, although the mechanistic basis was not established by this association.[47] Kochanek and colleagues evaluated the impact of *Adora1* knock-out (KO) in a murine model for experimental TBI and found that KO mice develop lethal status epilepticus following TBI, suggesting a role for ADORA1 in preventing PTS.[48] Activation of ADORA1 seems to inhibit the microglial response following TBI, which may indicate that ADORA1 and its associated pathways represent a therapeutic option for TBI.[49] These findings not only suggest new treatment modalities for PTS, but may help identify patients more likely to benefit from PTS prophylaxis. Darrah and colleagues investigated the glutamic acid decarboxylase 1 and 2 (GAD1/GAD2), which catalyze the conversion of glutamate to GABA. In a study of 257 adults with severe TBI, they found that *GAD1* rs3828275 (CT,TT) was associated with higher risk for PTS (Odds Ratio: 5.6, 95%CI: 1.21, 25.90).[50] Apolipoprotein E (APOE) has been studied for its role in PTS, and some investigations have suggested an association between *APOE* ϵ4 homozygotes and PTS, but this finding has not been consistent.[51, 52] Variability in outcome measures, statistical power, patient characteristics, and methodologies

seems to be the driver of this variability; however, a systematic review by Lawrence and colleagues found that over 63% of studies investigating APOE $\epsilon 4$ was detrimental to outcomes post-TBI.[53]

1.2.1.2 Sedation and Analgesia

In the intensive care setting, sedation is used to minimize patient discomfort and control intracranial pressure (ICP) in patients with TBI.[12] Preferred agents for sedation in adults with TBI include propofol and midazolam due to their favorable effects on cerebral metabolism, ICP, and cerebral perfusion pressure (CPP).[54, 33, 55] Midazolam is metabolized by cytochrome P-450, family 3, sub-family A, member 4/5 (CYP3A4/5) enzymes. The *CYP3A5**3 haplotype is associated with decreased midazolam metabolism, but its effect may only be clinically relevant in the presence of concomitant moderate/strong CYP3A4 inhibitors (e.g. ketoconazole, clarithromycin).[56, 57, 58] Ketamine and dexmedetomidine may be used in some patients with TBI as adjunctive or solo agents due to decreased accumulation, lower risk for dependence, and/or ease with which patients can be awakened for neurologic assessments. Nevertheless, their use in routine clinical practice for TBI is less established, but their use has gained popularity.[33] Some studies assessing ketamine in for ICP control among individuals with severe TBI have suggested that it provides sedation with minimal impact on ICP, although this remains controversial.[59] Ketamine is primarily metabolized by CYP3A4, but at higher concentrations the metabolic contribution of cytochrome P-450, family 2, sub-family B, member 6 (CYP2B6) is more pronounced.[60] Li and colleagues found that *CYP2B6* (*6/*6) was associated with 59% and 40% decreased steady state clearance of ketamine compared to *CYP2B6* (*1/*1, *1/*6), respectively in a group of 49 adult patients.[61] Decreased clearance of ketamine may increase risk for ad-

verse events including adverse psychomimetic and cognitive reactions, and hepatic and/or renal toxicity.[61, 62] Variability in dexmedetomidine pharmacokinetics/pharmacodynamics may be explained by variations in its metabolic pathway through the cytochrome P-450, family 2, sub-family A, member 6 (CYP2A6) and UDP glucuronosyltransferase family 1 member A4 (UGT1A4), though no link has been found at this point. However, dexmedetomidine pharmacodynamics may be affected by variations in its target, the adrenoreceptor alpha 2a (ADRA2A).[63, 64] Yagar and colleagues found that *ADRA2A* rs1800544 (GG, GC) was associated with slightly decreased efficacy at some time points as measured by sedation scores, though the clinical relevance of this finding is not clear.[65] Barbiturates, particularly pentobarbital, are also used as sedatives for TBI patients with refractory intracranial hypertension.[12]

TBI patients may also receive therapy with opioid analgesics/sedatives like fentanyl, remifentanil, or morphine.[33] Variants in *ABCB1*, *CYP3A5*, and the μ opioid receptor 1 (*OPRM1*) drive variable response to opioid medications commonly used for TBI.[66, 67] *ABCB1* rs1045642 (TT) is associated with decreased expression of ABCB1, which may increase penetration of ABCB1 substrates across the intestinal epithelium and the BBB. Fentanyl and morphine are substrates for ABCB1, and individuals carrying *ABCB1* rs1045642 (TT) may require lower doses. Lotsch and colleagues measured oral morphine equivalent dosing in subjects treated with ABCB1 substrates (e.g. morphine, fentanyl). They found that subjects with *ABCB1* rs1045642 (TT) required 135.4mg vs (CT) 194.9mg vs (CC) 274.5mg (ANOVA $p = 0.014$).[66, 68] Similarly, Horvat and colleagues found in a diverse pediatric population (N=61) that patients with *ABCB1* rs1045642 (TT) required 18.6mcg/kd/day less fentanyl than (CT,CC).[69] Patients with *CYP3A5**3 may also require a reduction in dose of fentanyl due to decreased hepatic clearance.[68] The *OPRM1* variation rs1799971 (GG) is associated

with decreased efficiency of endogenous opioid signaling.[70] *OPRM1* rs1799971 (GG) may predict increased dosing requirements for analgesics, and individuals with AG or AA genotypes may experience higher rates of adverse reactions.[67, 36]

1.2.2 Long Term Rehabilitation

Chronic neurologic and psychiatric care is necessary for many individuals post-TBI and pharmacotherapy may be guided by PGx. The use of PGx concepts to guide treatments for TBI rehabilitation in light of genetic risk factors and expected response to medications complements the *rehabilomics* framework introduced by Wagner in 2010.[71] Rehabilomics in the context of TBI refers to systematic use of biomarker, genetic data, phenotype data, and other patient specific factors that impact rehabilitation and long-term recovery.[71, 72] It focuses on TBI as a chronic disease state that requires long term care with patient-specific approaches to rehabilitation. An example of this is found in the approach by Myrga and colleagues in evaluating the sex-stratified risk for post-TBI cognitive decline in association with dopamine pathways. They found an important sex * gene interaction in patient outcomes, suggesting sex associated stratification of genetic risk.[73] While rehabilomics is all-encompassing, PGx is most applicable in the psychiatric and neurologic pathologies requiring pharmacotherapy such as post-traumatic depression (PTD), cognitive decline, and post-traumatic epilepsy (PTE).[74, 72] PTD and changes in cognitive capacity are multi-factorial, but incidence, nature and onset, as well as severity and duration may be predictable through genetic risk factors associated with monoamine pathways, specifically the dopamine (DA) pathways in the pre-frontal cortex and by serotonin pathways.[73] PTE may share some risk factors with PTS, but is thought to have unique pathophysiology.[75] Genetic influences on risk for these complications

are summarized in **Table 1.2**.

1.2.2.1 Post-Traumatic Depression

Post-traumatic depression occurs in up to 50% of individuals in the first year after TBI.[91] Selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) are frequently prescribed antidepressants, yet require up to six weeks to have an effect and have a poor response rate (the first choice only works in about 50% of individuals).[92] This necessitates a trial-and-error approach to antidepressant selection, which can result in patients waiting several months before finding an optimal therapy. Understanding if and how genetics moderates premorbid psychiatric disease relationships to PTD risk is an important consideration. The 5-HTTLPR variation is a tandem repeat in the promoter region for the serotonin transporter gene; *SLC6A4*, which is defined as either long (L) or short (S). L-homozygotes for this variation were found by Failla and colleagues to be at nearly three-fold higher risk for PTD, which may help to select patients who should be treated with antidepressants (e.g. SSRIs).[89]

Genetic predictors of risk for depression may be augmented by published PGx guidelines for selection and dosing of SSRIs when genetic data are available. Decreased doses of citalopram, escitalopram, and sertraline are recommended in cytochrome P-450, family 2, sub-family C, member 9 (CYP2C19) poor metabolizers. Citalopram and escitalopram are not recommended in CYP2C19 ultra-rapid metabolizers. Paroxetine and fluvoxamine are metabolized by cytochrome P-450, family 2, sub-family D, member 6 (CYP2D6). Paroxetine is not recommended in ultra-rapid metabolizers or poor metabolizers at CYP2D6, and a 25-50% dose reduction for fluvoxamine is recommended in poor metabolizers at CYP2D6.[93] Information about depression risk and drug metabolism may help clinicians appropriately monitor pa-

Table 1.2: Drug-Gene Pairs for Traumatic Brain Injury

Gene	HG38*/Common Name	rsID	Genotype(s)	Complication	References
ABCB1	NC_000007.14:g.87509329A>G	rs1045642	AA	Unclear**	[76, 77]
ABCC1	NC_000016.10:g.16144637G>A	rs4148382	GG	Improved six month outcomes (GOS* Scores)	[76]
ABCC8	NC_000011.10:g.17440757A>C	rs2283261	CC	Higher risk for cerebral edema	[78]
	NC_000011.10:g.17465190C>T	rs3819521	TT	Higher risk for cerebral edema	[78]
	NC_000011.10:g.17451890C>T	rs2283258	TT	Higher risk for cerebral edema	[78]
ADK	NC_000010.11:g.74683339A>G	rs11001109	GG	Increased seizure durationshorter time to first seizure	[79]
ADORA1	NC_000001.11:g.203163914A>G	rs3766553	AA	Higher risk for post-traumatic seizures	[47]
	NC_000001.11:g.203139380T>C	rs10920573	CT	Higher risk for post-traumatic epilepsy	[47]
	NC_000001.11:g.203163914A>G	rs3766553	AA	Higher risk for post-traumatic epilepsy	[47]
ANKK1	NC_000011.10:g.113400106G>A	rs1800497	GA	Higher composite cognitive score	[80]
APOE	Epsilon 4	NA	$\epsilon 4/\epsilon 4$	Higher risk for post-traumatic seizures	[51, 52]
AQP4	NC_000018.10:g.26855854C>T	rs3763043	TT	Unfavorable six month outcomes (GOS* Score)	[81]
	NC_000018.10:g.26865469T>C	rs3875089	CT;CC	Poor six month outcomes (GOS* Score)	[81]
COMT	NC_000022.11:g.19963748G>A	rs4680	GA;GG	Higher risk for cognitive decline	[82, 83, 84, 85]
DRD2	NC_000011.10:g.113410351G>C	rs6279	GG;GC	Higher composite cognitive score	[80]
GAD1	NC_000002.12:g.170826230C>T	rs3828275	TT	Higher risk for post-traumatic seizures	[50]
	NC_000002.12:g.170852920A>G	rs769391	AA	Higher risk for post-traumatic epilepsy	[50]
	NC_000002.12:g.170815681G>T	rs3791878	GG	Higher risk for post-traumatic epilepsy	[50]
IL-1β	NC_000002.12:g.112832813G>A	rs1143634	AG	Higher risk for post-traumatic epilepsy	[86]
MTHFR	NC_000001.11:g.11796321G>A	rs18001133	TC;TT	Higher risk for post-traumatic epilepsy	[87]
NT5E	NC_000006.12:g.85465856G>A	rs9444348	GA	Increased seizure durationshorter time to first seizure	[79]
SLC1A1	NC_000009.12:g.4557296C>G	rs10974620	GG	Higher risk for post-traumatic epilepsy	[88]
SLC6A4	5-HTTLPR	NA	Long/Long	Higher risk for post-traumatic depression	[89]
VMAT2	NC_000010.11:g.117265701G>C	rs363226	GC;GG	Higher risk for cognitive decline	[90]

HG38: Human Genome Version 38; **: Conflicting data; NA: Not Applicable

tients and to select appropriate pharmacotherapy early post TBI.

1.2.2.2 Post-Traumatic Cognitive Decline

Post-traumatic cognitive decline is common in patients with severe TBI and is thought to be driven by white matter loss (i.e. progressive damage to axonal tracts) and hippocampal atrophy.[94] While some patients' cognitive function improves in the immediate year following TBI, over five million Americans suffer from life-long progressive cognitive decline from TBI.[94] Genetic predictors may provide insight into the heterogeneity for risk and severity of cognitive decline.[29] They may also provide insight into what patients will benefit from early treatment. Effective pharmacotherapy for cognitive decline from TBI is not currently available and its development presents unique challenges. This is evident by a Cochrane review by Dougall and colleagues, which concluded that there is insufficient evidence for the effectiveness of pharmacotherapy for cognitive decline post TBI, however; their inclusion only evaluated modafinil, atomoxetine, rivastigmine, and an investigative monoamine modulator.[95] Additional study into other agents for cognitive decline is warranted.

Failla and colleagues evaluated the role of the dopamine receptor D2 gene (*DRD2*) and its genomic neighbor; ankyrin repeat and kinase domain containing 1 (*ANKK1*) in 99 Caucasians with severe TBI. They found that *DRD2* rs6279 (GG,GC) was associated with higher (improved) composite cognitive score at six months and *ANKK1* rs1800497 (GA) was associated with higher composite cognitive score at six and 12 months post TBI.[80] Wagner and colleagues investigated the role of the WW domain-containing protein (WWC1) in 129 patients with severe TBI for its impact on memory. They found that *WWC1* rs17070145 (CC) was associated with improved performance on episodic memory tests.[96] The rs363226 (GC,GG) genotypes in the vesic-

ular monoamine transporter 2 gene (*VMAT2*) was identified by Markos and colleagues to be associated with increased risk for cognitive decline as measured by cognitive-composite T scores. VMAT2 takes up monoamine neurotransmitters from the cytosol to vesicles, where they are stored for later release into the synapse.[90] In Catechol-O-methyltransferase (*COMT*) rs4680, the (GA,GG) genotypes are associated with numerous cognitive impairments after TBI, including worse nonverbal cognitive performance, PTSD, worse self-reported behavior among survivors with PTD, and worse executive functioning in pediatric TBI.[82, 83, 84, 85]

Beyond its utility as a predictor of outcomes, variations in dopamine pathways are associated with response to stimulants, anti-psychotics, and others.[97] Pharmacotherapy with methylphenidate has shown benefits for post-TBI cognitive impairment, and increased knowledge of how genetics influences various elements of cognitive performance that are amenable to improvement with a particular pharmacological intervention may guide cognitive testing, medication selection, and follow-up and care post-injury.[98]

1.2.2.3 Post-Traumatic Epilepsy

Post-Traumatic Epilepsy is defined by the occurrence of unprovoked seizures that occur seven days or more after TBI. The five year risk for PTE is 11.5% for people with severe TBI, and 1.6% for those with moderate TBI.[75] Genetic markers may predict risk for PTE and guide therapeutic decisions. Diamond and colleagues evaluated both Interleukin 1β (IL-1β) levels and associated genetic variations in the *IL-1β* gene with risk for PTE development. They found that the *IL-1β* rs1143634 (AG) genotype was associated with nearly three-fold increased risk or PTE and higher cerebro-spinal fluid (CSF)/serum ratios of IL-1β.[86] The methylenetetrahydrofolate reductase (*MTHFR*) gene has been

investigated for its role in epilepsy, including PTE, as MTHFR dysfunction can lead to elevated homocysteine and lowered seizure threshold.[99, 87] Scher and colleagues investigated the role of MTHFR in a population of from the armed forces with evidence of epilepsy diagnosis and history of TBI. They found that *MTHFR* rs18001133 (TC,TT) genotypes were associated with higher risk for PTE when they limited their population to subjects with two or more encounters for epilepsy (Adjusted Odds Ratio: 2.55).[87] Similar to the associations of the adenosine pathway and PTS, *ADORA1* rs10920573 (CT) and rs3766553 (GG) are associated with increased risk for PTE.[47] Also in that pathway; adenosine kinase (*ADK*) rs11001109 (GG), and ecto-5'-nucleotidase (*NT5E*) rs9444348 (GA) are associated with increased seizure duration and shorter time to initial seizure in patients who develop PTE.[79] In addition to their work in PTS, Darrah and colleagues found that *GAD1* rs769391 (AA) and rs3791878 (GG) were associated with PTE risk between one to six months post TBI.[50] Ritter and colleagues found that *SLC1A1* rs10974620 (GG) and rs7858819 (TT) were associated with higher risk for PTE.[88]

The use of genetic markers to predict incidence of PTE may help clinicians monitor and treat patients with PTE more effectively. Importantly, prophylactic treatment with anti-epileptic drugs does not reduce the incidence of PTE.[100] Genetic variations may have contributed to failure of randomized controlled trials and may have a role in supporting future drug development. PGx may also have similar application to the long-term use of phenytoin in relation to CYP2C9 metabolizer status, or the decision to not use it based on presence of HLA-B*57:01.[37, 38]

1.3 PHARMACOGENOMICS-GUIDED DRUG DEVELOPMENT

Drug development for TBI has focused on better understanding of the variables most associated with response. These include biometric measures (e.g. height, weight, age), type of injury, co-occurring diseases, and genetic factors. Evaluating genetics of TBI in association with disease progress and clinical outcomes has identified numerous areas for targeted drug development.[29, 101] In the very active area of TBI drug development, many efforts have focused on the prospect of drug re-purposing, often a much more affordable and easily translated approach.[31] This approach allows for fast clinical translation due to the decreased need for pre-clinical development and safety trials.[31]

1.3.1 Sulfonylurea Medications

Development of cerebral edema occurs in nearly half of patients with severe TBI, particularly in patients with computerized tomography (CT) evidence of mass lesion.[102] Prevention and treatment strategies include decompressive craniectomy, decreasing ICP through CSF drainage, and/or hyperosmolar therapy with mannitol or hypertonic saline.[12] The ATP-binding-cassette, subfamily C, member 8 (ABCC8) has received attention recently due to its association with the transient receptor potential melastatin 4 (TRPM4) specifically in the traumatically-injured brain. TRPM4 permits the transcellular flux of Na+, which leads to ionic edema (i.e. driven by fluid high in sodium and low in protein). ABCC8 regulates TRPM4 by closing the channel when intracellular ATP is high.[103] The ABCC8 antagonist, glyburide (glibenclamide), is thought to prevent the opening of the ABCC8-TRPM4 channel post-TBI. It is under investigation as a treatment for cerebral edema post TBI, has been successful in pre-clinical studies, has shown

promise in clinical trials in stroke, and is currently in phase II clinical trials for prevention of CE in moderate/severe TBI (clinical trials identifier: NCT01454154).[31, 104]

Jha and colleagues studied ABCC8 with a candidate gene approach for risk of developing cerebral edema (CE) post severe TBI. They found significant associations with rs2283261 (CC), rs3819521 (TT), and rs2283258 (TT) in the *ABCC8* gene with increased risk of CE following severe TBI (OR: 2.45, 2.95, and 3.00, respectively).[78] This finding suggests a genomic role wherein ABCC8 PGx may help predict the occurrence of CE, and may help guide the appropriate use of glyburide in the intensive care unit. An additional contributor to risk for CE post-TBI is Aquaporin-4 (AQP4).[105] Dardiotis and colleagues investigated the role of AQP4 with clinical outcomes following TBI and found that *AQP4* rs3763043 (TT) and rs3875089 (C) were associated with a higher odds of having an unfavorable and favorable six-month GOS scores, respectively, which suggests that a molecular moderator of CE risk (AQP4) impacts TBI outcomes.[81] These findings support the important role of pharmacologically protecting from CE in TBI patients. Glyburide, among other sulfonylurea medications, is also metabolized by the polymorphic CYP2C9.[37] While there are no current clinical recommendations regarding dosing when used as an anti-diabetic agent, glyburide is given at a sub-therapeutic dose in TBI relative to its FDA-approved dosing for diabetes. Hypoglycemia is undesirable post TBI, and it is possible that CYP2C9 poor/intermediate metabolizers may be more likely to develop hypoglycemia.[103, 37]

1.3.2 Modulation of CNS Metabolism

Cytochrome p-450 enzymes (CYPs) are also present in the brain and in the cerebrovascular endothelium.[106] Little has been investigated regarding the role of genetics of brain-specific CYPs, but Donnelly and colleagues investigated CYP polymor-

phisms in patients with subarachnoid hemorrhage (SAH). They found that *CYP4A11* rs9332978 (CT,CC) was associated with decreased CSF of the cerebral vasoconstrictor, 20-hydroxyeicosatetraenoic acid (20-HETE). Results also showed that the GG genotype at rs3093089 in the *CYP4F2* gene was associated with decreased risk for clinical neurologic deterioration. Finally, they found that *CYP4A11* rs3890011 (GC,CC), *CYP4F2* rs3093156 (TA,TT), and *CYP4F2* rs3093168 (AA) were associated with higher odds of having a favorable outcome.[107] Further Investigations are needed to delineate how brain specific CYPs affect drug disposition in the context of TBI. This area has been an important focus in drug development for SAH and may be an area of drug development for TBI with evidence of hemorrhage.[107]

1.3.3 CNS Antioxidants

Oxidative stress drives the secondary injury early post-TBI, and brain antioxidant reserve is depleted after severe TBI.[108, 109] CNS antioxidant use has been under investigation as a treatment strategy for TBI. N-Acetylcysteine is an FDA approved medication that is under investigation as a therapy for TBI to increase the brain concentration of glutathione (GSH), a potent CNS antioxidant.[31] Due to its hydrophilicity and poor penetrance through the BBB, it also is being investigated in combination therapy with the SLC22A6 and SLC22A8 inhibitor, probenecid.[110] This strategy has been shown to increase brain concentrations of NAC in pre-clinical studies, and might increase its efficacy.[111]

1.4 THE ROLE OF TRANSPORTER PROTEINS IN TRAUMATIC BRAIN INJURY

Previous sections have discussed the roles of diverse genes that are related to TBI recovery, outcomes, and complications. Transporter genes are of particular interest post TBI because 1) they support the movement of helpful and toxic solutes in and out of the brain and 2) their impact in either facilitating or blocking the entry of medications into the brain.[112] These are loosely defined in two super-groups, the ATP-Binding Cassette (ABC) transporters and the solute-carrier (SLC) transporters. The ABC transporters are exclusively dependent on ATP hydrolysis to transport solutes from the intracellular space to the extracellular space, often counter to concentration gradients.[112] Depending on localization, they may move solutes across apical or basolateral membranes. The SLC transporters are more frequently dependent upon concentration gradients of co-substrates, often sodium gradients. They are most often uptake transporters, but some are efflux or bi-directional.[112] Both classes of transporters are drivers of active transport throughout the body and are critical to the maintenance of concentration gradients.

1.4.1 Transporters as Mediators of Traumatic Brain Injury

Transporters are expressed on the BBB, blood-CSF barrier, neurons, and microglia. They mediate the entry of impermeable substrates, and move toxic substrates from the CNS to the blood so that they can be eliminated.[113] Recent work has demonstrated that genetic variation in transporters are associated with outcomes following TBI. Cousar and colleagues investigated genetic variations in BBB transporters (ABCB1, ABCC1, and ABCC2) in 305 adult patients with severe TBI. They found that *ABCB1* rs1045642 (AA) and *ABCC1* rs4148382 (GG) genotypes were associated with

lower odds of unfavorable six month Glasgow Outcome Scale (GOS) scores, defined as GOS of 1-3 (Odds Ratio: 0.71 and 0.73, respectively).[76] The finding with *ABCB1* rs1045642 was reversed in a study by Wang and colleagues who studied 182 patients with TBI. They defined favorable outcomes as GOS of 3-5 and found that patients with the (AG,GG) genotypes were more likely to have favorable GOS scores at six months post TBI (Odds Ratio: 2.71)[77] These contradictory results may be due to different definitions of outcomes and/or racial composition (i.e. Caucasian vs. Asian). The ABC transporter, ABCG2, is closely related to ABCB1 and ABCC1 and is also highly expressed in the CNS. ABCG2 has not been studied in the context of TBI, but its role in removing substrates that may be relevant to pathophysiology in TBI (e.g. protoporphyrin IX and uric acid) suggest that it may play a similar role post TBI as ABCB1 and ABCC1.[114]

The discovery of ABCG2 was published in 1998 by Doyle and colleagues after it was found in the MC-7 breast cancer cell line as a contributer to efflux of anthracycline chemotherapeutic agents.[115] It was aptly named breast cancer resistance protein and investigated for its role in chemotherapy resistance in many cancers.[115] This led to investigations into the use of xenobiotics that inhibit ABCG2 as adjunctive cancer therapy in addition to chemotherapy.[116] A similar strategy has been employed for other transporters with roles in drug resistance. Targeted pharmacologic modification of transporter function in the brain may be therapeutic for TBI. This is exemplified by the work by Hagos and colleagues by using the transporter inhibitor, probenecid, to promote brain concentrations of N-Acetylcysteine.[111]

1.4.2 Knowledge Gaps for Transporters in TBI

Despite the growing body of literature suggesting that transporters are relevant and important to brain recovery post TBI, little is known about what drives these individual effects. Answering these questions requires a focused approach that targets individual transporters of interest with a focus on mechanistic relationships with TBI pathophysiology and recovery. These can be studied in a clinical/translational manner by investigating transporter response to injury in experimental TBI (e.g. gene expression), measuring clinical outcomes in relation to functional genetic variation in transporters, and studying the impact of transporter dysfunction on substrate disposition. Very little is known about how transporter gene expression changes after TBI in various brain tissues. Study into this area would complement investigations into the association of functional transporter variations with injury, thus providing a reasonable pathway into new drug development pathways and clinical translation.

1.4.3 The Role of ABCG2 Following TBI

The ABC transporter, ABCG2, is of particular interest within the area of neurologic disease due to its high level of expression in the brain and potential role in removing xenobiotics and endogenous substrates from the brain.[117] It is highly expressed in humans and animals and has roles in maintenance of endogenous substrates and xenobiotics. ABCG2 is also polymorphic, with high frequency functional variations that are conducive to clinical study.[118, 119]

1.4.3.1 ABCG2 Regulation, Structure, and Function

The *ABCG2* gene spans approximately 66 kilobases with 16 exons.[120] The pro-

moter region begins approximately 400bp upstream from the first exon, which lacks TATA and CCAAT boxes.[120] This *ABCG2* promoter region contains contains multiple binding sites for the SP1, SP3, AP1, AP2, and XBBF transcription promoters.[120] Upstream transcription regulation for *ABCG2* is driven by the Aryl Hydrocarbon Receptor (AHR),[121] the hypoxia-inducible factor complex 1 (HIF-1)[122], the estrogen receptor (er),[123] nuclear factor erythroid 2-related factor 2 (NFE2L2),[124] peroxisome proliferator-activated receptor gamma (PPARγ)[125], and the progesterone receptor (PR).[126] Baseline expression of *ABCG2* is likely maintained by activation of AHR, and induced in the presence of environmental toxicants.[127] This is particularly noted in the induction of AHR by tobacco smoking, which induces the transcription of *CYP1A2* and *ABCG2*.[127] This suggests that increased *ABCG2* transcription is beneficial in the presence of environmental toxicants.[127]

Inhibition of *ABCG2* expression on the BBB is associated with cytokines, particularly interleukin-6 (IL-6), interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α).[128] MicroRNAs 200c, 212, 328, 519, and 520 have also been found to inhibit expression of *ABCG2*.[129] The precise mechanism by which cytokines inhibit expression of *ABCG2* mRNA has not been fully elucidated. The mechanism for inhibition of *ABCG2* transcription from cytokines is likely not related to the JAK/STAT downstream pathway from IL-6, as the *ABCG2* gene does not contain the binding site.[120] Cytochrome P-450 expression inhibition by IL-6 is driven by the downstream transcription factor C/EBPβ-LIP, but this binds the CCAAT box, which is not present on the *ABCG2* gene.[130, 131] This suggests a more indirect link between cytokine signaling and inhibition of *ABCG2* gene expression.

The ABCG2 protein is a 72-kDA membrane transport protein that is phylogenetically related to other ABC transporters, most closely ABCG1 and only distantly re-

lated to ABCB1.[132] The "G" family of ABC transporteres are referred to as reverse transporters due to the reversed positioning of the nucleotide binding domain with respect to the N-terminus.[132] ABCG2 is expressed as a half transporter and is thought to form homo-oligmers in order to properly function.[133] ABCG2 effluxes a diverse range of substrates including xenobiotics; uric acid (UA); and porphyrins, such as protoporphyrin IX. Within the brain, ABCG2 is expressed in endothelial cells of the BBB, ependymal cells of the blood-CSF barrier, microglia, and neurons.[134, 135] On the BBB, it is expressed on the apical membrane and faces blood removing its substrates from the brain compartment, thus playing a primary role in both limiting CNS exposure and removing molecules generated within the CNS .[134] This directionality is reversed on the blood-CSF barrier, where it faces CSF on choroid plexus ependymal cells.[135] This suggests a potential inverted relationship between the concentration of ABCG2 substrates in the brain versus the CSF, with respect to plasma concentrations (**Figure 1.2**).

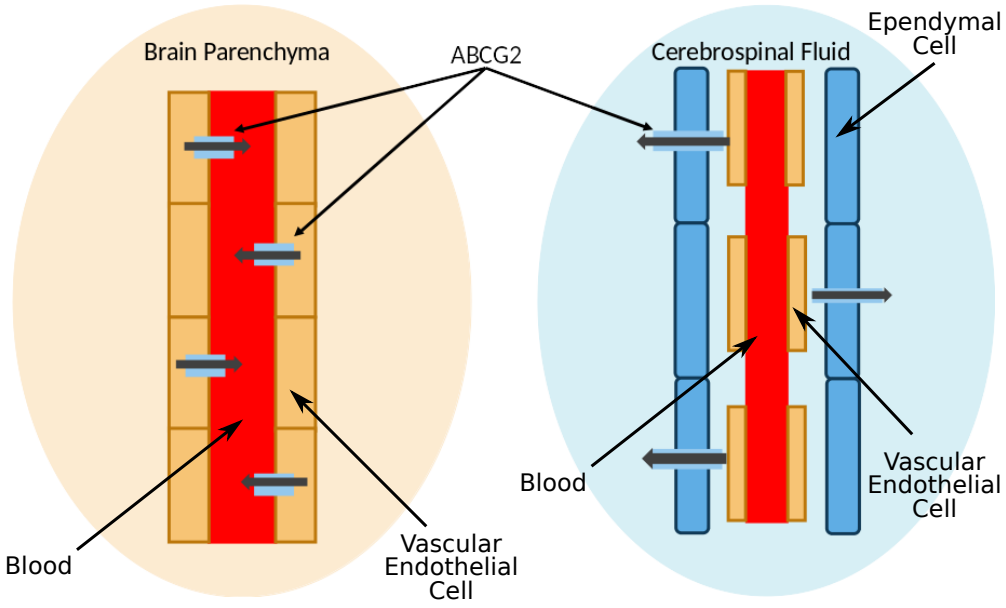

Figure 1.2: ABCG2 protein expression is blood (red) facing on the blood brain barrier (right) and CSF facing on the bood-CSF barrier (left). ABCG2 substrates may exhibit an inverse relationship with respect to ABCG2 expression changes in the CSF versus the brain.

1.4.3.2 ABCG2 Genetic Variation

Common functional genetic variations in the *ABCG2* gene are found in most populations. Minor allele frequencies (MAFs) for the non-Finnish European population as reported in the gnoMAD browser are used throughout this project.[136] Two missense variations are particularly, including rs2231137 (c.34G>A, p.Val12Met, MAF=0.0409) and rs2231142 (c.421C>A, p.Gln141Lys, MAF=0.1036).[136] Extensive work by Tamura and colleagues has found that the c.421C>A variation is associated with normal mRNA expression and decreased protein expression.[119] They also found that c.34G>A is associated with normal mRNA and protein expression and possibly increased trans-

porter function, as measured by survival of transfected cells treated with cytotoxic substrates of ABCG2.[119] The presence of two common and functional variations that can predict both increased and decreased transporter activity/function provide an ideal scenario for studying the role of ABCG2 following TBI.

1.4.3.3 ABCG2 and Uric Acid in Neurologic Disease

The c.421C>A variation in the *ABCG2* gene is of particular interest in neurologic diseases due its impact on the transport of UA. Individuals who carry at least one "A" allele have higher risk for hyperuricemia and gout due to decreased renal elimination of UA.[137] Uric acid is interesting on its own due to its role as an antioxidant, and that has led to substantial interest in studying the role of rs2231142 and UA in neurologic diseases associated with increases in oxidative stress. Matsuo and colleagues investigated rs2231142 in in patients with Parkinson's disease and found that patients carrying at least one variant allele developed Parkinson's disease later, suggesting a protective effect.[138] UA alone has also been implicated as a protective agent against Lewy Body Dementia.[139] Feher and colleagues noted similar findings in Alzheimer's disease, where the variant "A" allele was protective against the disease.[140] It is not known if c.34G>A is associated with an opposing effect on UA transport or disease risk.

1.5 CONCLUSION

Traumatic brain injury research is aimed at attaining better understanding of the disease processes and developing novel treatments. The use of PGx to support drug development has also been supported by the myriad genetic association studies, which

have identified several common genetic variations that are associate with disease. Associations between genetic variation in transporter genes and TBI outcomes has highlighted the growing role for transporters as markers of disease and targets for treatment.

The work described in this dissertation focuses on the role of transporters in TBI. We are interested in the potential association of genetic variations in the *ABCG2* gene in patients suffering from severe TBI. Furthermore, we seek to further explain changes in brain chemistry that occur as a result of severe TBI within the pediatric and adult brain.

1.6 HYPOTHESIS

The current knowledge of TBI with respect to transporters suggests that several genes that are already understood to impact drug disposition might have a role in recovery following TBI. This generates further interest because these are already under investigation for their role in cancer drug resistance and drug-drug interactions. We are particularly interested in studying the disposition of transporters following injury, and specifically focusing on the role of the ABC transporter, ABCG2, due to its potential role in removing cytotoxic porphyrins and UA from the brain.

The objective of this work is to determine the genetic and molecular role of the transporter, ABCG2, following severe TBI. We hypothesize that 1) transporter expression is globally stunted following severe TBI due to increase in inflammatory cytokines and 2) decreased function of ABCG2 as predicted by genetic variation is associated with improved clinical outcomes following severe TBI due to increased CNS concentra-

tion of UA. These hypotheses are tested in the following aims:

Aim 1: Measure the Timing and Magnitude of Transporter Expression Changes Following Experimental Traumatic Brain Injury

This study will investigate the time-dependent changes in transporter expression in experimental TBI. It will utilize a pre-clinical model of pediatric severe TBI.

Aim 2: Evaluate the Role of ABCG2 genetic variation on Clinical Outcomes Following Severe TBI

This aim will consist of a retrospective clinical cohort (discovery and replication) trial evaluating the impact of genotype on longitudinal outcomes.

Aim 3: Measure the Impact of Uric Acid Disposition on Clinical Outcomes Following Severe TBI

This study will evaluate the levels of UA in the CSF and plasma in patients with severe TBI in contrast to TBI outcomes and ABCG2 genotype. It will utilize a subset of the subjects sampled in aim 2.

2.0 TRANSPORTER EXPRESSION FOLLOWING EXPERIMENTAL PEDIATRIC TRAUMATIC BRAIN INJURY

2.1 INTRODUCTION

Growing knowledge of TBI suggests that transporters are relevant prognostication and treatment targets. However, little is known about how the changes following TBI impact transporter expression. In this section, we sought to determine the timing and magnitude of expression changes in transporters following TBI.

2.1.1 Role of the CNS Barriers in Traumatic Brain Injury

Key to the progression of secondary injury are changes in the BBB, blood-CSF barrier, and permeability of neurons and microglia. This is driven by physical changes from injury, complex biochemical perturbations, which include changes in expression of transporters on the BBB and within the brain.[26, 141] This change in transporter expression changes the brain's ability to uptake nutrients, remove toxins, and may have substantial effect on the flux of pharmacological agents across the BBB, neurons, and microglia.

2.1.2 Previous Studies of Transporters and Gene Expression in TBI

Traumatic brain injury has dramatic effects on the neuro-chemical milieu, with varying directionality and magnitude depending on the time following the primary injury.[54] An investigation by Willyerd and colleagues investigated protein expression for the ATP-Binding cassette transporters ABCB1 and ABCC1 in post-mortem human brain tissue and found that ABCC1 protein levels in cortex of injured subjects was higher than control, and that ABCB1 showed little change.[141] However, this study combined diverse sampling times (based on time from injury) that is not able to comprehensively evaluate temporal differences in expression. Transcriptomic profiling post TBI

has been carried out by Di Pietro, et al. using a stretch model for TBI in organotypic hippocampal slice cultures. They identified several pathways involved in secondary injury that were associated with inflammation and cytokines.[142] These findings suggest that transporter expression changes due to the pathophysiology of TBI, and that those changes may be important to understanding the progression of secondary injury after TBI.

2.1.3 Objective and Hypothesis

Little is known about the directionality, timing, and magnitude of transporter expression changes in the pediatric brain. Answering these questions will increase the understanding of secondary injury and will aid in the identification of molecular targets for secondary injury.[143] There is also limited information regarding the relative levels of the expression of different transporters at baseline in the pediatric brain and poor understanding of the changes associated with development. We sought to 1) develop a targeted panel of transporter genes in the brain, 2) determine baseline relative transporter expression in pediatric and adult rats, 3) determine the magnitude and timing of expression changes following experimental TBI, and 4) correlate changes in transporter expression with activated pathways. We hypothesized that transporter mRNA expression would be decreased following experimental TBI in conjunction with enrichment of pathways associated with inflammation and hypoxia.

2.2 METHODS

2.2.1 Gene Expression Panel

We developed a targeted panel of transporters of potential relevance to pathophysiology of secondary injury and/or drug development. First, we conducted a literature review to identify transporters, biomarkers, and transcription factors that were of probable importance to TBI pathophysiology. This was judged based on 1) expected expression in the brain, 2) previously noted roles in brain disease, and 3) role in transport for medications used in TBI. Next, we added transporters that are recommended for evaluation in the context of drug-development by the International Transporter Consortium and FDA guidelines.[144] Biomarkers and transcription factors were added to the panel based on 1) evidence for a role in TBI and 2) data supporting their role in modulating expression of genes related to metabolism. Finally, those human genes were transposed to their corresponding rat homologues by using the NCBI HomoloGene. Housekeeping genes were selected based on evidence for stability post TBI. The final list of genes was developed into a custom panel for quantitation of mRNA using the nCounter® (NanoString Technologies, Seattle, WA) platform. This technology allows for the absolute quantitation of transcripts using barcoding, which enables deeper comparisons between genes and tissues.

2.2.2 Animal Husbandry

All animal experiments were performed in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Charles River, Wilmington, MA) were used for this experiment. Rats were housed in a temper-

ature (22°C) controlled room with a 12h light/dark cycle and allowed access to water and chow *ad libitum* during the experiment, and were weighed daily. Seventy-two post natal day (PND) 17 rats were randomized 2:1 to experimental TBI and sham injury. Eight PND 17 rats and four adult rats (300g) remained naïve to injury for a total of 84 rats. Animal groupings and time of sacrifice were randomized *a priori* to control for litter effects.

2.2.3 Experimental TBI

Experimental TBI was carried out using the controlled cortical impact (CCI) model.[145] Rats were anesthetized with a 2:1 mixture of N_2O:O_2 with 4% isoflurane for induction and 2% for maintenance through a nosecone. Continuous rectal temperature was measured and maintained at 37°C using a heated pad. The head was secured using a stereotaxic frame with ear pins. The scalp was shaved and prepared with a betadine swab. Using sterile aseptic technique, a mid-line sagittal incision was made followed by reflection of the scalp with retractors. A high speed air-powered dental drill was then used to make a 7mm craniotomy in the left parietal bone, which was removed to expose the dura. A calibrated pneumatic piston was then used to impact the intact dura with a 2.5mm deformation and impact velocity of 4m/s. Sham injured animals received the full procedure with the exception of the impact.

2.2.4 Dissection

CCI and sham rats were sacrificed at 3, 12, 24, 72, 168, and 336h post-surgery. Naïve PND 17 rats were sacrificed at PND 17 and at PND 31. Adult rats were sacrificed upon arrival to our facility. All work surfaces and surgical tools were cleaned with

RNaseZap® (Thermo Fisher Scientific, Waltham, MA) before each procedure. Rats were placed in a plastic chamber and subjected to 4% isoflurane and 2:1 N_2O:O_2 for induction of anesthesia. Once anesthetized, rats were transferred to an absorbent pad and affixed with a nosecone providing 2% isoflurane with 2:1 N_2O:O_2 for maintenance of anesthesia. Following cardiac puncture and withdraw of blood (for plasma), rats were decapitated, and the brain was extracted and placed on an inverted glass petri dish on a bed of wet ice. Blood was injected into a 1.5mL microcentrifuge tube pre-loaded with 5μL of heparin, then centrifuged at 2500G for 5 minutes, then the plasma was pipetted into a fresh microcentrifuge tube and immediately frozen on liquid nitrogen. The brain was rinsed gently with ice cold normal saline frequently during dissection. The ipsilateral and contralateral cortices were removed first, followed by the ipsilateral and contralateral hippocampi. Finally, the choroid plexus was removed with pointed, curved forceps. After processing the brain, the abdomen was dissected and a single kidney was removed and an approximately 50g section of liver tissue was removed. Each tissue sample was placed in a separate, pre-labeled microcentrifuge tube, then immediately frozen with liquid nitrogen and later stored at -80°C until further processing.

2.2.5 Isolation and Analysis mRNA and Protein

This study also presented an opportunity to build a repository of animal tissue for future study. As such, we were interested in methods that facilitated high quality sample collection, storage, and processing. These methods are summarized below and focus on development of methods specific to the expected downstream applications of sample analysis.

2.2.5.1 Method Development

To facilitate current and future applications of the tissue collected from this study, we sought to develop a method to obtain high quality RNA and protein. Initially, we employed a method suggested by a Qiagen supplementary protocol, which suggested using 4 volumes of ice-cold acetone to the initial RNeasy column filtrate, centrifuging, then reconstituting.[146] This was initially tested using brain tissue from a previous rat study for the integrity of the protein. We compared protein collected through acetone precipitation with crude cell lysate loaded on an sodium-dodecyl-sulfate poly-acrylamide gel electrophoresis (SDS-PAGE), then with western blotting. We used Abcg2 antibody as a probe given that previous experience in our lab showed that antibody affinity for Abcg2 protein is sensitive to sample processing. The acetone precipitation sample showed strong signal for the β-actin band, but a virtually absent Abcg2 band. The lysate western blot showed strong signal for Abcg2 and β-actin, suggesting that Abcg2 was either lost in the acetone precipitation step, or that the other chemicals involved in the Qiagen RNeasy procedure caused sufficient damage to the Abcg2 protein to prevent the antibody from binding. Finally, we developed a compromise method in which microcentrifuge tubes containing frozen whole tissue (hippocampus, cortex, choroid plexus) were filled with an aliquot of radioimmunoprecipitation assay (RIPA) buffer containing 1% v/v beta-mercaptoethanol and homogenized using a hand-held homogenizer. A 150μL aliquot of the homogenate in RIPA buffer was used in place of the typical starting material in the manufacturer's protocol. We tested the quality of mRNA in this procedure as well using a bio-analyzer to ensure that mRNA was not damaged in the modified procedure **Figure 2.1A**. The remaining homogenate was frozen at -80°C and was evaluated with SDS-PAGE and western blot to determine Abcg2 presence **Figure 2.1B**.

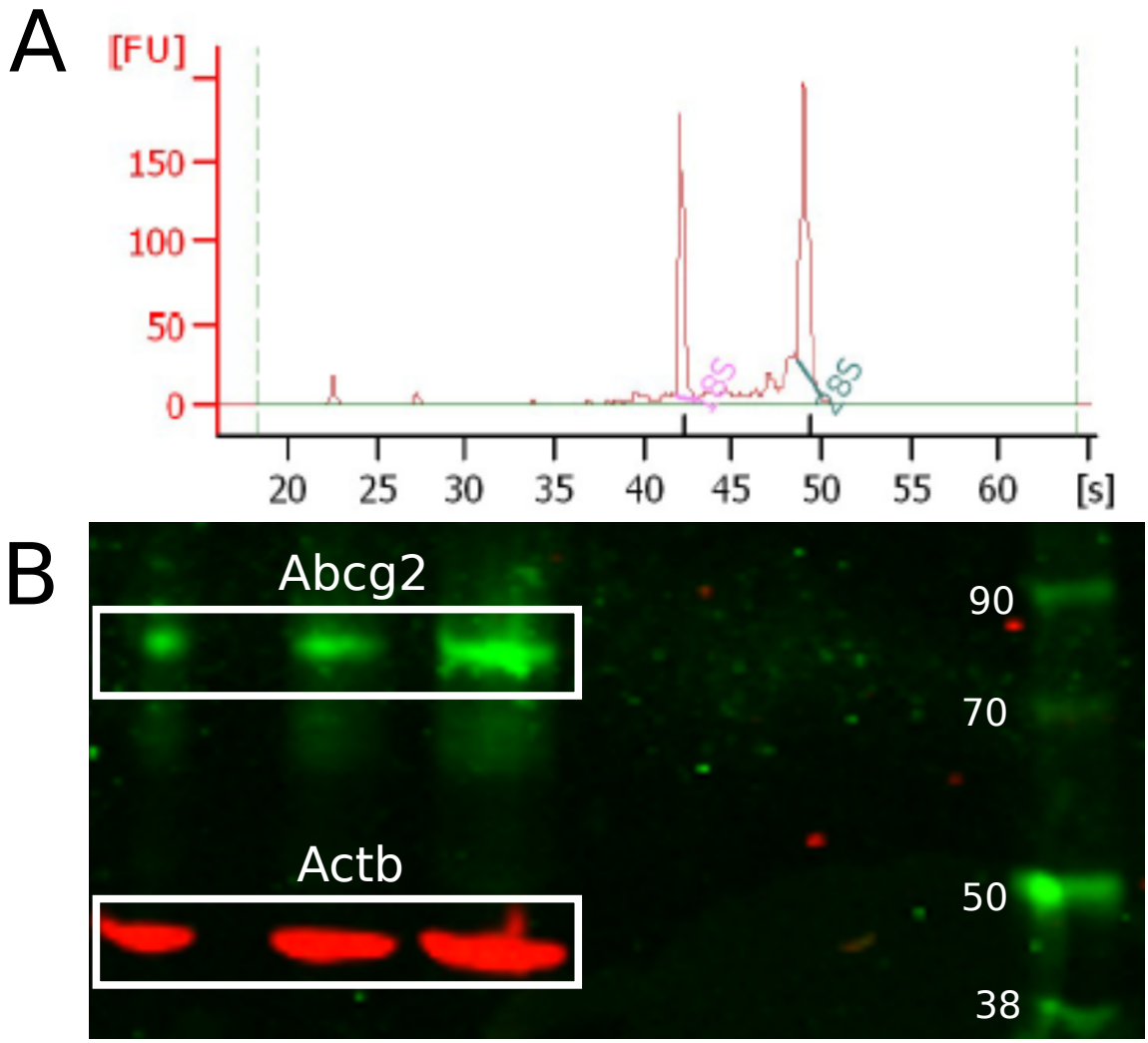

Figure 2.1: Results of western blotting and bioanalyzer to verify protein and mRNA quality. Figure 2A depicts a representative plot of bioanalyzer output for mRNA collected through our modified Qiagen protocol. Few small peaks and sharp peaks at the 18s and 23s ribosomal subunits suggest that mRNA is relatively intact (i.e. high quality). Figure 2B depicts a crude western blot verifying the presence of antibody-targetable Abcg2 and beta-actin protein.

2.2.5.2 Tissue Processing and mRNA Isolation

mRNA was isolated from tissues using a modified version (see previous section) of the manufacturer's protocol with the RNeasy Mini Kit (QIAGEN, Hilden, Germany). For liver and kidney, the standard Qiagen procedure was used after manually homogenizing the liver and kidney tissue with a mortar and pestle while frozen at -80°C. Approximately 30g of the pulverized liver and kidney was used for mRNA isolation with the remaining placed in a microcentrifuge tube and frozen at -80°C. For the choroid plexus, we also used the standard Qiagen procedure and loaded th entirety of the tissue collected into the microcentrifuge tube for homogenization. RNA quality and concentration were assessed with NanoDrop (Thermo Fisher Scientific, Waltham, MA).

2.2.6 Measurement of mRNA Expression with Nanostring

2.2.6.1 Selection of Samples

Samples were selected based on 1) previously planned sample inclusion and 2) extra slots available on gene expression panel that could potentially answer supplementary questions. Pre-determined tissues include 1) naïve hippocampus and cortex in PND 17, PND 31, and adults; 2) naïve choroid plexus, liver, and kidney in PND 17 and PND 31; and 3) all sham and CCI ipsilateral hippocampus and cortex. Due to an additional availability of nine slots on the plate, a subset of four CCI rats had mRNA expression measured in the contralateral hippocampus, and five contralateral cortex from the 24h post-CCI time point. The samples were then diluted with RNase/DNase free water to a concentration of 30ng/μL to satisfy the 3μL loading volume for Nanostring with a goal starting amount of approximately 100ng mRNA. mRNA quantification was conducted using the manufacturer's instructions for Nanostring. Briefly, capture probes with a biotinylated tail and reporter probes with a fluorescent barcode com-

plementary to target transcripts are hybridized with 100ng of each sample at 65°C overnight, then loaded on the Prep Station®, which is an automated liquid handler that removes excess sample and unbound codesets in a two-step bead clean-up protocol. After purification, probe/transcript complexes are eluted and transferred to the streptavidin coated nCounter® cartridge, which binds the biotinylated region of the probe/transcript complex. Next, a current was applied to align the reporter probes. The cartridge was then scanned with a Digital Analyzer, which scans 600 fields of view across each cartridge and counts the occurrence of each fluorescent barcode. Digital counts are then reported for normalization and analysis.

2.2.7 Statistical Methods

Data normalization and summary statistical analyses were carried out using R v3.4.3 (R Development Core Team, Vienna, Austria).

2.2.7.1 Nanostring Data Normalization

Raw transcript counts were obtained from the nCounter® platform and normalized with the R package NanoStringNorm. The variation was assessed for all possible combinations of normalization parameters using the following code.

```
require(NanoStringNorm)
RCC_Files <- read.markup.RCC(rcc.path = "RCC/") #Reads files
compare_norms <- norm.comp(RCC_Files) #Compares normalization methods using
   signal to noise
```

To remain conservative, we did not utilize the "OtherNorm" options for normalization, which include variant stabilization and normalization for micro-array data (VSN), z-score normalization, or quantile normalization. While these methods can further optimize the variance, they are not well documented or accepted for Nanostring data. We

also opted, based on the default normalization method, to limit to geometric mean for the code-count normalization with a strategy of minimizing the variance in code count, housekeeping, and end result variation. Despite not minimizing the variance, we used the mean alone, rather than the mean plus two standard deviations as the correction for background noise. This provides a more liberal consideration for whether signal is from actual mRNA or from noise (i.e. fewer samples considered zero expression). This was due to the high quality mRNA provided, relatively few mRNA targets, and expectation that all, or almost all, of the targets would be present in each sample. The normalization parameters, as coded, are demonstrated in the following code.

```
Data_Norm <- NanoStringNorm(RCC_Files,
                      CodeCount = "geo.mean",
                      Background = "mean", #Note that mean.2sd provides
                         better variance, but more low expression samples as
                         "0"
                      SampleContent = "housekeeping.geo.mean",
                      OtherNorm = "none",
                      take.log = TRUE,
                      return.matrix.of.endogenous.probes = FALSE
                      )
```

Samples with a background value greater than three standard deviations from the mean were re-run if possible, or excluded from further analysis. Following normalization, all counts were log2 transformed for downstream analysis. These steps were separated for all tissues evaluated (i.e. cortex, hippocampus, liver, kidney, choroid plexus). Analyses were completed in two sets in 2015 and 2017, with slight modifications to the panel in 2017. This required separate normalization for tissue batches that were then merged. Normalization findings for variation compared across methods are included in **Appendix A**.

2.2.7.2 Analysis of Naïve Data

Counts from naïve animals at PND 17, PND 31, and adults in hippocampus and cortex were measured to determine differences associated with development. Data for PND 17 and 31 was also measured in choroid plexus, liver, and kidneys. For all tissues, relative expression compared to liver and kidneys was evaluated and presented as:

$$f(x, y) = \log_2(x) - \log_2(y);$$

where x and y are expression in the relevant brain tissue and liver or kidney, respectively. All pairwise comparisons for naïve data were not performed due to the volume of data that would be produced. Count data are presented as raw counts and as relative expression to liver and kidney. Raw count data for all naïve tissues are presented in **Appendix A, Tables A3 and A4**.

2.2.7.3 Analysis of CCI v. Sham

To measure changes associated with injury, transcript counts in the ipsilateral hippocampus and cortex were compared with sham animals at each time point with unpaired t-tests. Counts for injured rats were compared with sham rats at each time point for fold change. Fold change P values were corrected using Benjamini-Hochberg False Discovery Rate (FDR) to produce q values.[147] For all analyses, a q-value of 0.05 was considered statistically significant. To identify enriched disease pathways and upstream regulators, log2 fold change and FDR values were input to Ingenuity Pathway Analysis (IPA) web-based software (QIAGEN, Hilden, Germany) with a FDR q-value cutoff of 0.1 for inclusion in analysis. Tissues considered were CNS tissue (not otherwise specified), cortex, and hippocampus. Enriched disease pathways and predicted upstream regulators were filtered from a comparison analysis across all six time points for each tissue with an activation z score absolute value > 0.

2.2.7.4 Analysis of CCI Ipsilateral vs. Contralateral

Comparison of ipsilateral vs. contralateral expression in CCI rats was carried out using a paired t-test. Fold change P values were corrected using Benjamini-Hochberg False Discovery Rate (FDR).[147] A q-value of 0.05 was considered statistically significant.

2.3 RESULTS

2.3.1 Gene Expression Panel Design

The literature review identified 27 rat transporters corresponding to 24 human homologues **Table 2.1**. To provide a limited physiological context for changes in expression, we selected four transcription regulators known for their roles in injury and in modulation of transporter expression (Nfe1l2, Hif1a, Nr1i2, Il6). To serve as validation and to ensure adequate control for the model, we added five biomarkers (Edn1, Gfap, Vim, Ngb, Icam1) for injury that are well characterized post injury, and four housekeepers to normalize the expression (Ppia, Gapdh, Tbp, Hprt1).

Table 2.1: Genes and Associated Homologues in Gene Expression Panel

HGNC ID	Human Homologue	Common Name
	Transporters	
Abcb11	*ABCB11*	BSEP
Abcb1a	*ABCB1*	MDR1/PGP
Abcb1b	*ABCB1*	MDR1/PGP
Abcc1	*ABCC1*	MRP1
Abcc2	*ABCC2*	MRP2
Abcc4	*ABCC4*	MRP4
Abcc5	*ABCC5*	MRP5
Abcc8	*ABCC8*	SUR1
Abcg2	*ABCG2*	BCRP
Slc15a1	*SLC15A1*	PEPT1
Slc15a2	*SLC15A2*	PEPT2
Slc16a1	*SLC16A1*	MCT1
Slc22a2	*SLC22A2*	OCT2
Slc22a4	*SLC22A4*	OCTN1
Slc22a6	*SLC22A6*	OAT1
Slc22a8	*SLC22A8*	OAT3
Slc28a2	*SLC28A2*	CNT2
Slc29a1	*SLC29A1*	ENT1
Slc2a1	*SLC2A1*	GLUT1
Slc47a1	*SLC47A1*	MATE1
Slc7a1	*SLC7A1*	CAT1
Slc7a5	*SLC7A5*	LAT1
Slco1a1	—	OATP
Slco1a2	—	OATP
Slco1a5	*SLCO1A2*	OATP1A2
Slco1b2	*SLCO1B3*	OATP8
Slco2b1	*SLCO2B1*	OATP2B1
	Transcription Factors	
Hif1a	*HIF1-A*	HIF1-A
Nfe2l2	*NFE2L2*	NRF2
Il-6	*IL-6*	IL-6
Nr1i2	*NR1I2*	PXR
	Biomarkers	
Gfap	*GFAP*	GFAP
Vim	*VIM*	VIM
Icam1	*ICAM1*	CD54
Ngb	*NGB*	NGB
Edn1	*EDN1*	ET1

2.3.2 Expression in Naïve Rats

Baseline expression of transporters in the naïve rats was similar across the hippocampus and the cortex. Overall, Slc2a1, a glucose transporter, was the most prominently expressed in the cortex and hippocampus with no evidence of differential expression associated with development. Multiple transporters in the organic anion transport protein (OATP) group of transporters (Slco1a2, Slco1a5, Slco1b2) showed differential expression in the hippocampus associated with development. Among these, only Slco1a2 was differentially expressed with development in the cortex. The peptide transporter, Slc15a1, showed differential expression in the cortex and hippocampus with a drop to near zero expression occurring from PND 31 to adulthood. Slc16a1, a lactate and pyruvate transporter, was of similar expression levels to Slc2a1 in PND 17 rats, with decreases in expression from PND 17 to adulthood in hippocampus and cortex. From PND 17 to Adulthood, 14 and 11 transporters showed differential expression associated with development in the hippocampus and cortex, respectively. With few exceptions, the majority of these changes were associated with differential expression from PND 17 to adult, suggesting the the PND 31 time point is an expression level intermediate within rat development. ABC transporters as a whole showed diverse expression patterns with modeest changes with development. Among these, transporters within the MRP group (Abcc2, Abcc4, and Abcc5) showed changes with development in the hippocampus.

Expression patterns compared to liver and kidney were also presented to contextualize the magnitude of transporter expression. Naïve expression for liver and kidney is presented in **Figures 2.2 and 2.3**, respectively. Cortex baseline expression and expression relative to liver and kidney are shown in **Figures 2.4 and 2.5**, respec-

tively. Hippocampus baseline expression and expression relative to liver and kidney are shown in **Figures 2.6 and 2.7**, respectively. Choroid plexus baseline expression and expression relative to liver and kidney are shown in **Figures 2.8 and 2.9**, respectively. Baseline data in naïve rats showed diversity among baseline levels of expression. Slc2a1 was the highest expressed transporter in all brain tissues. Relative to kidney and liver, all brain tissues expressed high levels of Abcc8, which had negligible expression in liver and kidney. Abc transporters

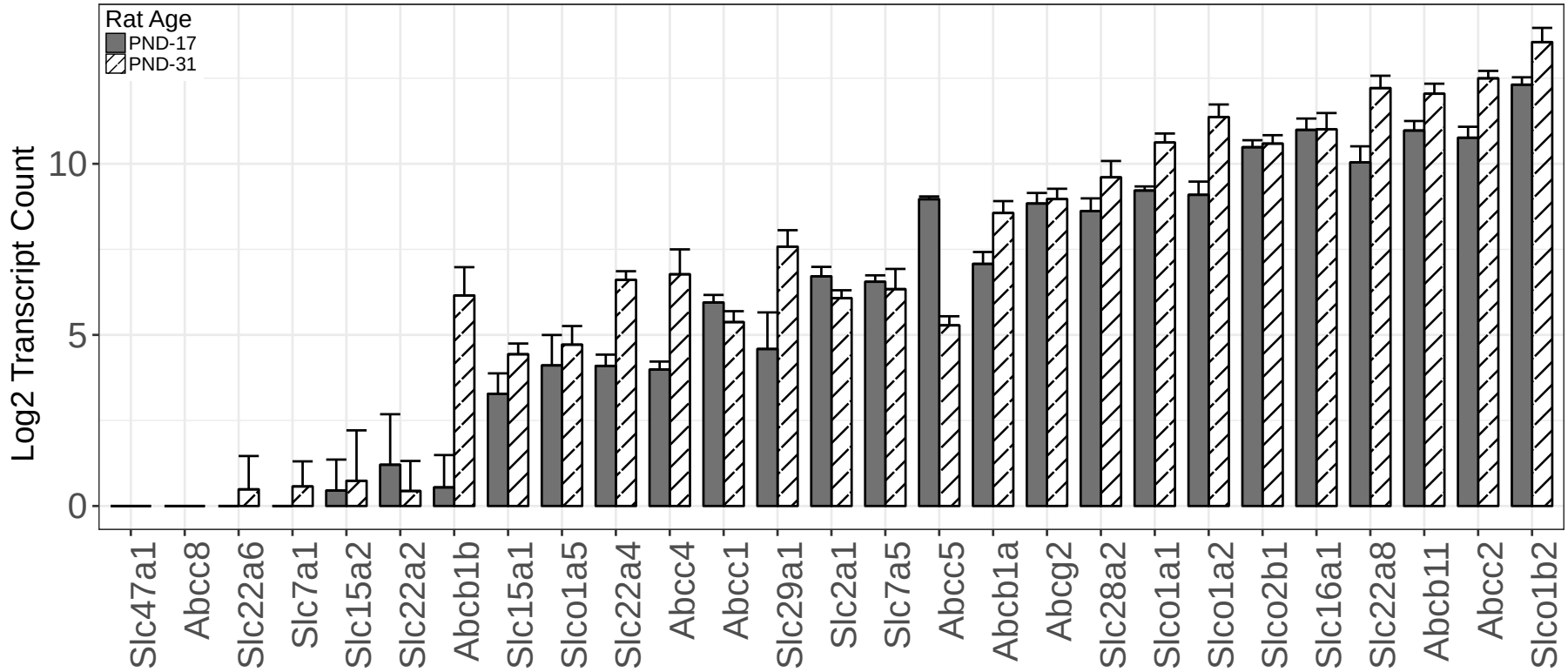

Figure 2.2: Baseline transporter expression in rat liver at PND-17 and PND-31.

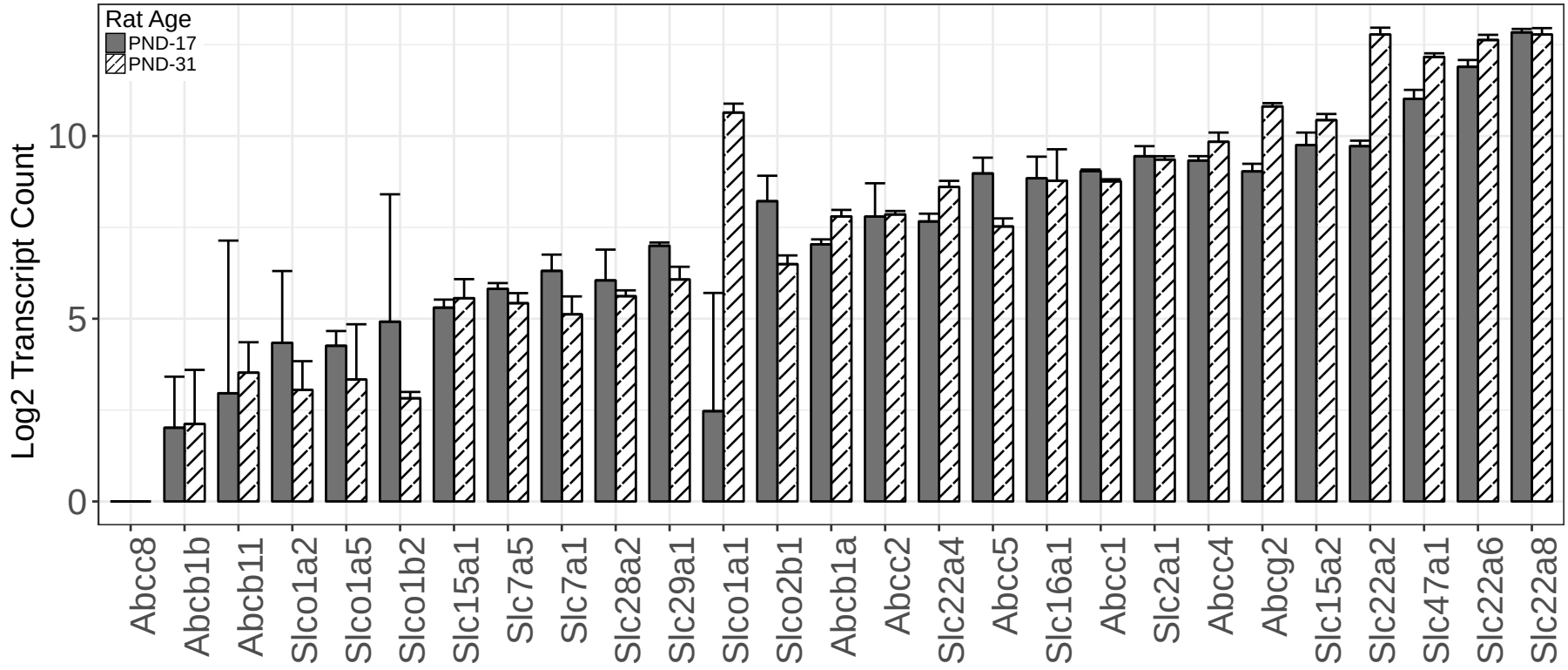

Figure 2.3: Baseline transporter expression in rat kidney at PND-17 and PND-31.

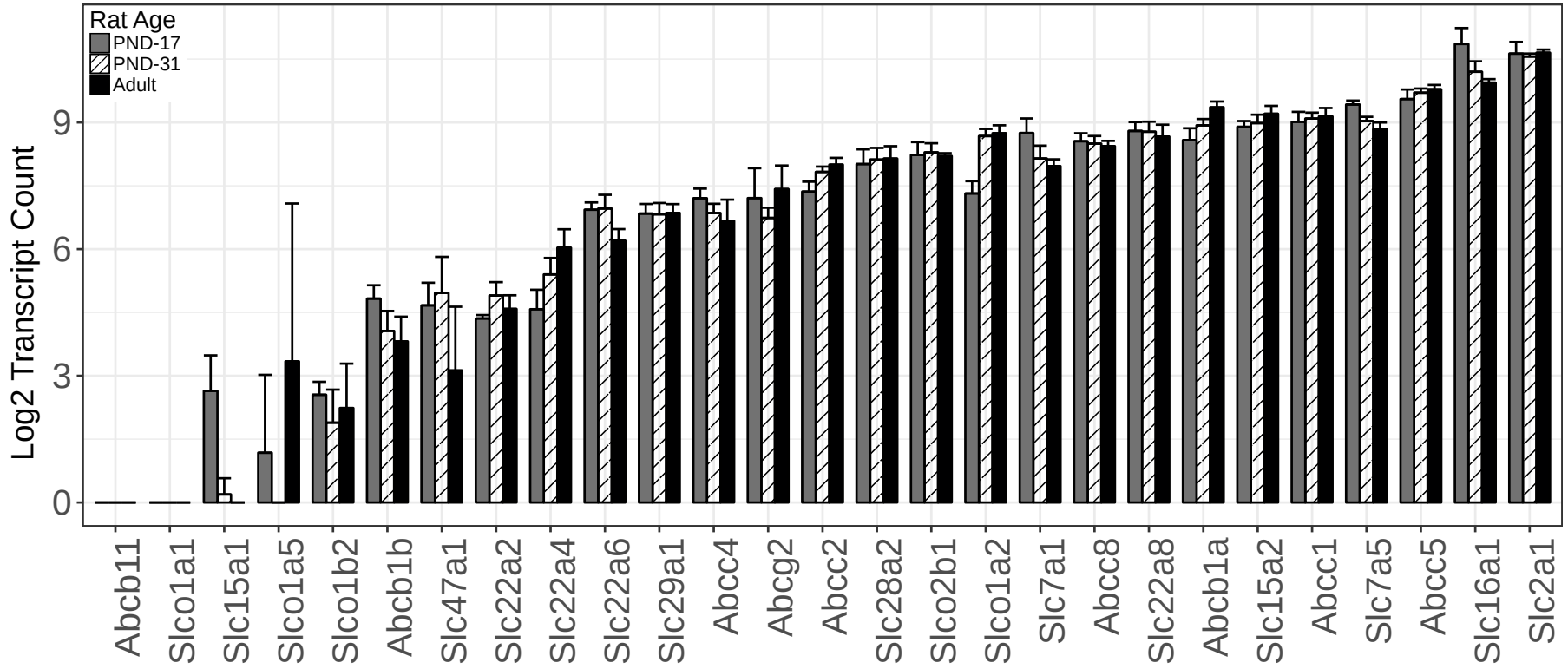

Figure 2.4: Baseline transporter expression in rat cortex at PND-17, PND-31, and adult.

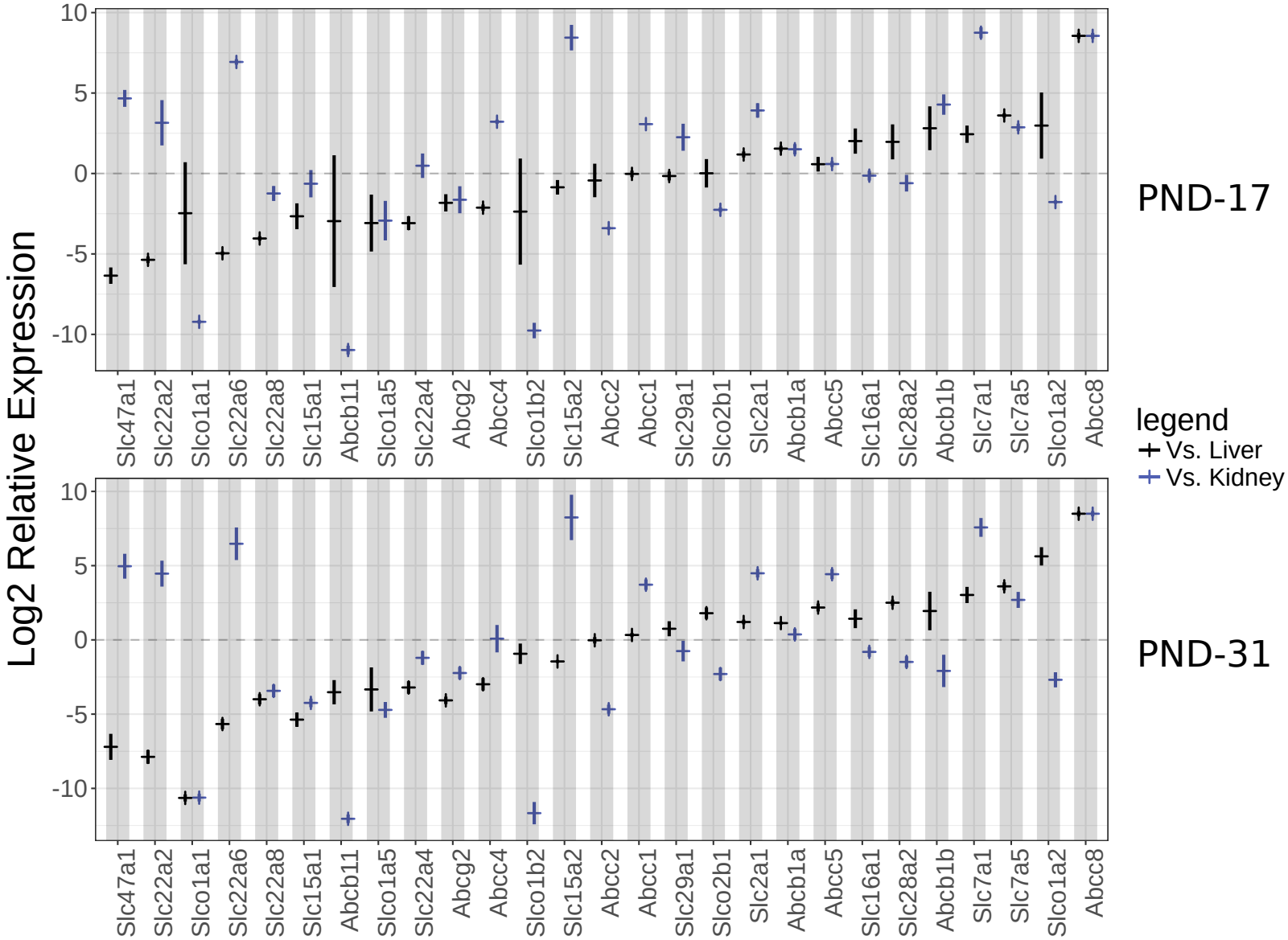

Figure 2.5: Naïve expression in rat cortex at PND-17 and PND-31 relative to liver and kidney. Statistical significance can be inferred from error bars (95% CI) that do not cross the zero line.

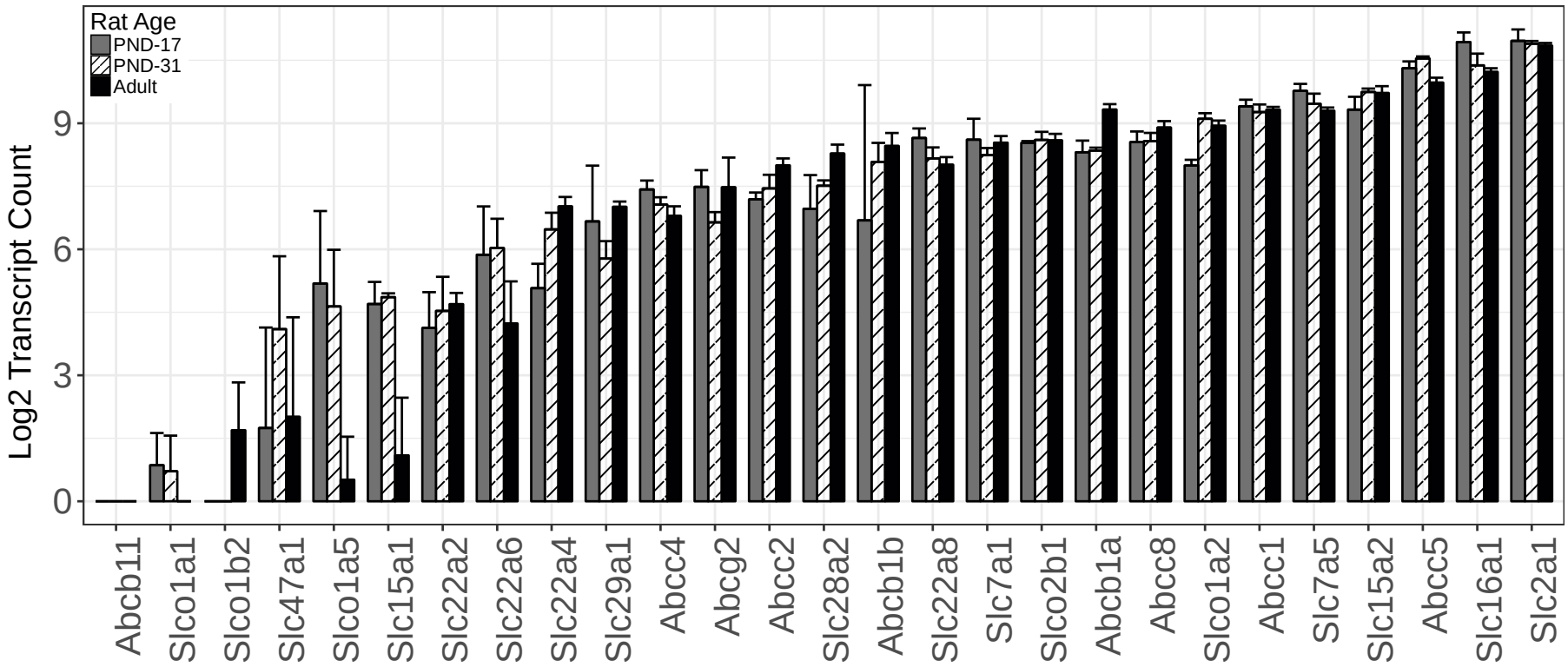

Figure 2.6: Baseline transporter expression in rat hippocampus at PND-17, PND-31, and adult.

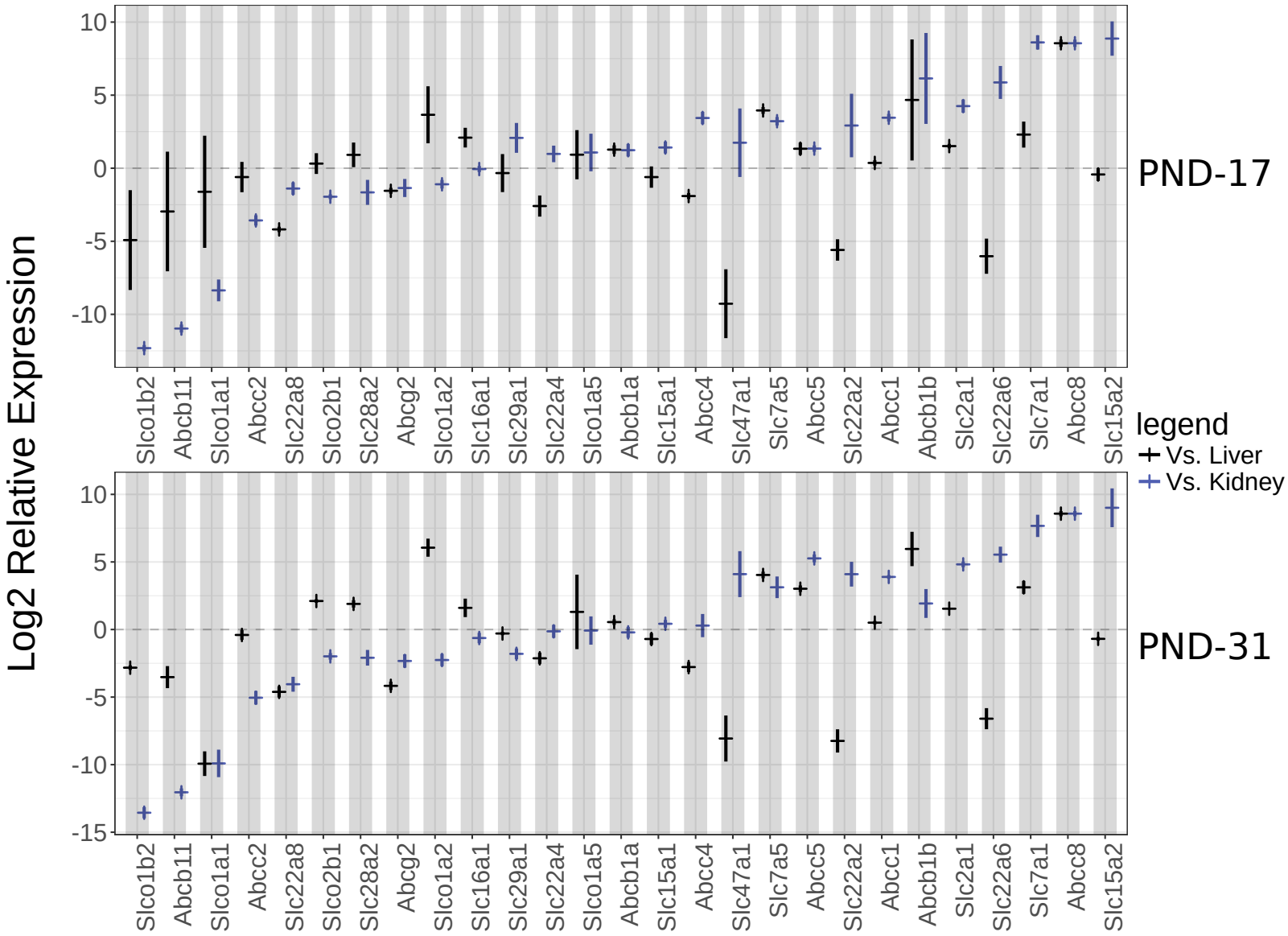

Figure 2.7: Naïve expression in rat hippocampus at PND-17 and PND-31 relative to liver and kidney. Statistical significance can be inferred from error bars (95% CI) that do not cross the zero line.

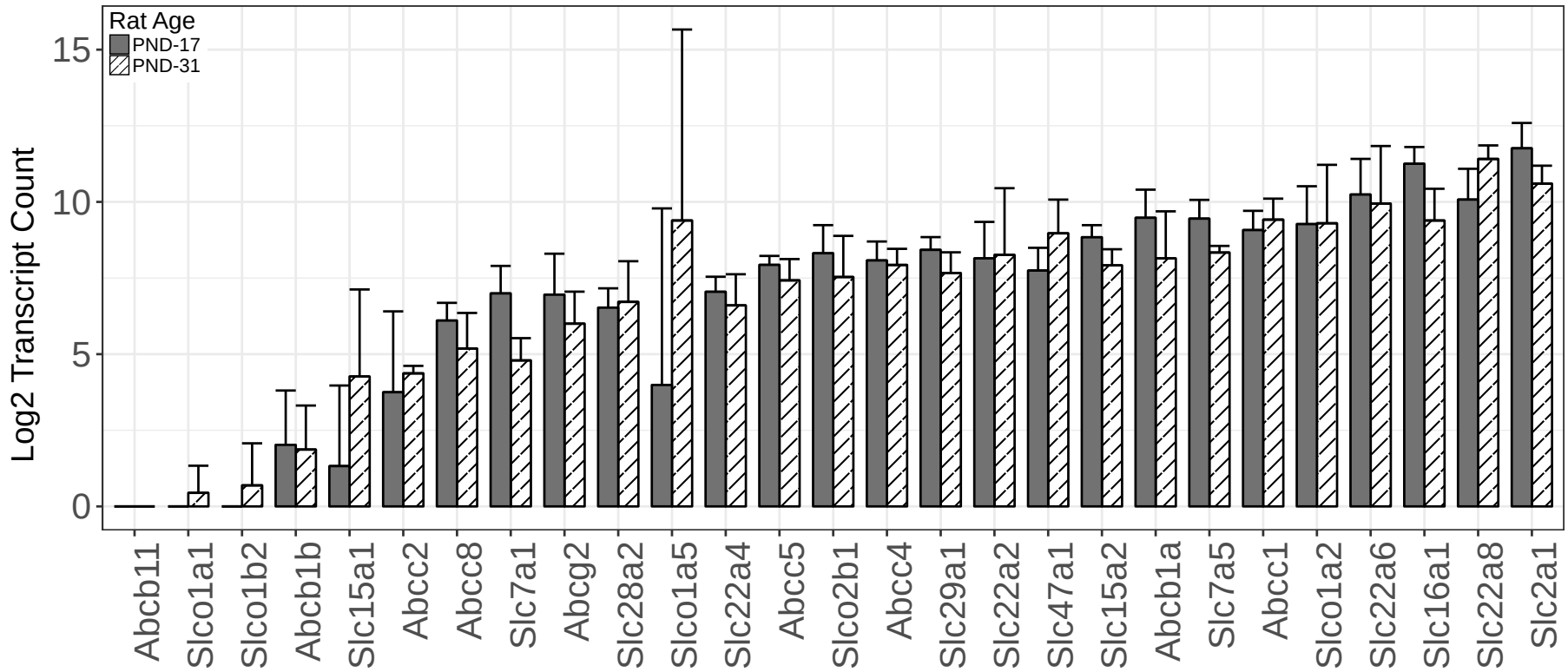

Figure 2.8: Baseline transporter expression in rat choroid plexus at PND-17 and PND-31.

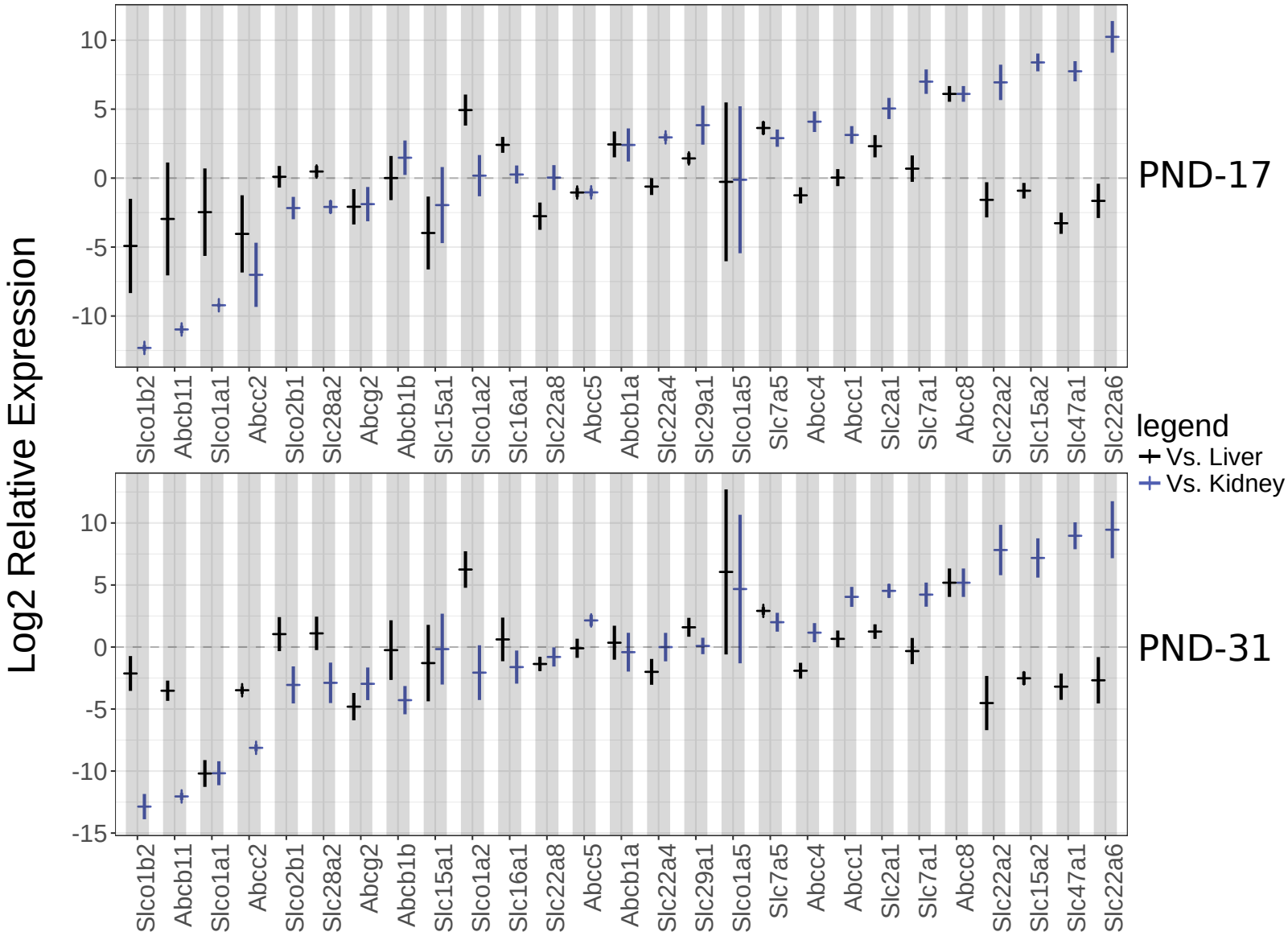

Figure 2.9: Naïve expression in rat choroid plexus at PND-17 and PND-31 relative to liver and kidney. Statistical significance can be inferred from error bars (95% CI) that do not cross the zero line.

2.3.3 Expression Changes Due to Injury

2.3.3.1 CCI vs. Sham

Eight out of nine ABC transporters showed significantly differential expression from sham in at least one time point in hippocampus **Table 2.2** and cortex **Table 2.3**. We found that changes in ABC transporters were mirrored in cortex and hippocampus in all cases, with some visually apparent variation in magnitude **Figure 2.10A**. ABC transporters exhibited either early down-regulation followed by gradual return to baseline, or delayed up-regulation. Interestingly, Abcb11 showed a spike in expression that was significant in the cortex. These patterns appear to follow patterns similar to Hif1a and Il6 **Figure 2.10D**. Similarly to ABC transporters, many SLC transporters showed an early decrease in expression followed by a gradual return to baseline or an inversion to over-expression **Figure 2.10B**. Biomarkers for TBI showed mostly predictable changes in expression. In contrast to expectations, we found significantly decreased expression for Ngb in both hippocampus and cortex **Figure 2.10C**.

Table 2.2: Gene Expression Changes in the Hippocampus

Gene	3 Hours		12 Hours		24 Hours		72 Hours		7 Days		14 Days	
	Log2 FC	FDR	Log2 FC	FDR	Log2 FC	FDR	Log2 FC	FDR	Log2 FC	FDR	Log2 FC	FDR
Abcb11	0	NA	0	NA	0	NA	0.809	0.12	0	NA	0	NA
Abcb1a	-0.634	0.01	-0.812	0.002	-0.22	0.114	0.03	0.933	0.039	0.887	-0.039	0.894
Abcb1b	-0.541	0.567	-1.034	0	-1.326	0	0.084	0.939	-0.194	0.415	-0.151	0.581
Abcc1	-0.114	0.582	-0.285	0.072	0.022	0.886	0.192	0.306	0.282	0.057	-0.004	1
Abcc2	-0.331	0.063	-0.49	0.056	-0.592	0.021	-0.336	0.169	-0.036	0.894	0.122	0.582
Abcc4	-0.227	0.453	-0.034	0.931	0.399	0.017	1.06	0.006	0.692	0.001	0.281	0.378
Abcc5	-0.069	0.38	-0.064	0.473	0.011	0.939	0.069	0.582	-0.07	0.451	-0.054	0.582
Abcc8	0.167	0.197	-0.154	0.197	-0.713	0.046	-0.761	0.011	-0.495	0.002	-0.343	0.222
Abcg2	-0.918	0.019	-0.924	0.093	-0.468	0.018	-0.405	0.17	0.056	0.931	-0.328	0.393
Edn1	1.256	0.006	0.239	0.577	0.838	0.005	0.124	0.708	0.15	0.554	-0.222	0.611
Gfap	0.704	0.008	2.038	0	2.351	0.001	2.836	0.001	1.916	0	1.539	0.006
Hif1a	0.407	0.003	1.024	0	0.939	0	0.536	0.042	0.171	0.006	0.284	0.392
Icam1	3.335	0	2.783	0.001	3.168	0.021	3.004	0	1.884	0	1.587	0.052
Il6	6.262	0.005	2.584	0.036	1.412	0.132	0.644	0.508	0	NA	0.869	0.509
Nfe2l2	0.836	0.001	0.896	0	0.773	0.001	1.798	0	1.148	0	0.893	0.027
Ngb	-0.123	0.822	-0.84	0.004	-1.211	0.006	-0.617	0.378	-0.103	0.769	-0.759	0.016
Nr1i2	0.181	0.7	-0.076	0.939	0.327	0.508	0.036	0.943	0.853	0.007	0.039	0.939
Slc15a1	-0.278	0.378	0.224	0.762	-0.037	0.897	0.199	0.563	0.213	0.499	0.163	0.582
Slc15a2	-0.279	0.004	-1.486	0	-1.954	0	0.431	0.064	0.543	0.004	0.469	0.034
Slc16a1	-0.037	0.894	-0.539	0	-0.351	0.008	0.692	0.036	0.619	0.003	0.594	0.07
Slc22a2	0.245	0.582	-0.208	0.582	-0.054	0.939	0.772	0.489	0.362	0.637	0.189	0.858
Slc22a4	-0.034	0.976	1.044	0.018	1.338	0.001	2.32	0	1.863	0	1.787	0.003
Slc22a6	0.227	0.508	-4.589	0.002	-3.646	0.009	0.102	0.939	0.164	0.897	0.991	0.433
Slc22a8	-0.337	0.266	-1.68	0.001	-1.442	0	-0.42	0.358	0.392	0.387	-0.322	0.231
Slc28a2	0.091	0.801	-0.536	0.088	0.664	0.128	1.502	0.013	-0.247	0.392	0.169	0.563
Slc29a1	-0.702	0.143	-0.793	0.002	-0.411	0.034	0.137	0.577	0.514	0.134	0.517	0.118
Slc2a1	0.457	0.079	0.614	0.001	0.664	0.007	0.546	0.058	0.331	0.063	0.438	0.051
Slc47a1	-0.608	0.763	-0.856	0.626	1.379	0.433	2.092	0.197	0.039	1	1.382	0.387
Slc7a1	-0.132	0.301	-0.192	0.378	-0.173	0.392	-0.189	0.563	-0.163	0.566	-0.257	0.244
Slc7a5	0.327	0.022	0.613	0.001	0.694	0	0.426	0.021	0.582	0.004	0.375	0.009
Slco1a1	0.058	0.984	0.469	0.305	0.796	0.168	-0.593	0.595	-0.367	0.784	-0.368	0.701
Slco1a2	-0.988	0.009	-1.54	0	-0.775	0.016	-0.189	0.577	0.105	0.621	0.108	0.554
Slco1a5	-1.362	0.433	-0.076	0.894	0.319	0.451	-0.155	0.118	1.715	0.231	-0.399	0.399
Slco1b2	0	NA	0	NA	0.121	0.508	0.021	0.508	0	NA	0	NA
Slco2b1	-0.61	0.022	-0.884	0	-0.594	0.002	0.688	0.001	0.968	0.001	0.741	0.024
Vim	0.485	0.017	1.881	0	3.909	0	5.091	0	3.286	0	2.609	0.005

Table 2.3: Gene Expression Changes in the Cortex

Gene	3 Hours		12 Hours		24 Hours		72 Hours		7 Days		14 Days	
	Log2 FC	FDR	Log2 FC	FDR	Log2 FC	FDR	Log2 FC	FDR	Log2 FC	FDR	Log2 FC	FDR
Abcb11	0.000	NA	0.170	0.479	1.149	0.068	1.837	0.019	1.357	0.043	0.769	0.197
Abcb1a	-0.187	0.583	-0.324	0.114	-0.145	0.673	0.243	0.114	0.415	0.032	0.275	0.293
Abcb1b	0.820	0.021	0.834	0.020	0.507	0.008	2.077	0.000	1.551	0.061	0.955	0.252
Abcc1	-0.180	0.333	0.090	0.405	0.321	0.003	0.421	0.013	0.269	0.070	0.189	0.410
Abcc2	-0.384	0.004	-0.505	0.063	-0.561	0.038	0.050	0.686	0.051	0.643	-0.224	0.292
Abcc4	0.186	0.401	0.156	0.377	0.260	0.207	0.775	0.004	0.597	0.004	0.474	0.270
Abcc5	-0.098	0.662	0.112	0.592	0.214	0.235	0.298	0.061	0.164	0.169	-0.009	1.000
Abcc8	0.267	0.039	0.025	0.937	-0.333	0.007	-0.231	0.019	-0.170	0.329	-0.284	0.372
Abcg2	-0.507	0.329	-0.619	0.158	-0.320	0.383	-0.267	0.214	0.074	0.852	-0.007	1.000
Edn1	1.166	0.003	1.232	0.002	0.823	0.097	0.300	0.401	0.611	0.212	-0.064	0.643
Gfap	0.411	0.092	1.768	0.000	2.156	0.020	2.699	0.002	2.495	0.004	1.834	0.051
Hif1a	0.247	0.042	0.753	0.002	1.029	0.000	0.277	0.013	0.234	0.111	0.276	0.237
Icam1	1.951	0.019	2.123	0.000	1.784	0.005	1.430	0.001	1.111	0.026	0.869	0.212
Il6	4.377	0.040	4.996	0.000	2.077	0.043	1.065	0.120	1.230	0.066	0.872	0.388
Nfe2l2	0.677	0.004	0.988	0.003	0.456	0.029	1.179	0.002	1.019	0.002	0.791	0.019
Ngb	-0.139	0.468	-0.841	0.005	-1.588	0.002	0.143	0.605	0.096	0.871	-0.379	0.247
Nr1i2	0.000	NA	0.105	0.479	0.000	NA	0.268	0.592	0.085	0.852	0.306	0.479
Slc15a1	0.241	0.765	0.058	0.954	-0.172	0.925	-1.023	0.173	-1.224	0.277	0.086	0.954
Slc15a2	-0.190	0.342	-0.932	0.000	-1.155	0.002	0.677	0.004	0.809	0.011	0.497	0.024
Slc16a1	-0.042	0.806	-0.448	0.003	-0.296	0.026	0.524	0.036	0.485	0.003	0.586	0.048
Slc22a2	-0.145	0.775	-0.524	0.198	0.027	0.966	0.248	0.490	0.456	0.212	0.021	1.000
Slc22a4	0.711	0.372	1.200	0.016	1.416	0.007	2.316	0.003	1.513	0.004	1.687	0.104
Slc22a6	-0.525	0.442	-2.825	0.015	-1.602	0.008	-0.060	0.933	1.452	0.092	0.505	0.583
Slc22a8	-0.208	0.563	-1.157	0.002	-1.493	0.000	-0.429	0.053	0.205	0.563	-0.141	0.726
Slc28a2	-0.543	0.029	-0.634	0.019	-0.066	0.927	0.508	0.114	-0.080	0.673	-0.290	0.479
Slc29a1	-0.261	0.120	-0.678	0.001	0.248	0.198	0.591	0.000	0.378	0.109	0.252	0.481
Slc2a1	0.394	0.087	0.789	0.002	0.705	0.030	0.085	0.643	0.301	0.137	0.393	0.070
Slc47a1	-2.303	0.036	-0.691	0.463	-1.280	0.210	-0.571	0.204	0.921	0.270	0.435	0.662
Slc7a1	0.076	0.705	-0.053	0.716	-0.157	0.270	-0.064	0.758	-0.007	1.000	0.061	0.726
Slc7a5	0.229	0.167	0.672	0.002	0.599	0.000	0.210	0.069	0.398	0.002	0.459	0.013
Slco1a1	0.000	NA	0.000	NA	0.000	NA	0.000	NA	0.000	NA	0.000	NA
Slco1a2	-0.479	0.277	-0.889	0.004	-0.647	0.197	-0.127	0.509	0.533	0.159	0.308	0.296
Slco1a5	-0.696	0.706	-1.346	0.293	0.105	0.627	0.699	0.383	-0.516	0.827	-0.055	1.000
Slco1b2	0.327	0.784	-0.517	0.563	0.318	0.827	0.254	0.792	-1.715	0.013	-0.155	0.927
Slco2b1	-0.268	0.377	-0.637	0.002	-0.490	0.003	0.716	0.004	1.203	0.001	0.712	0.132
Vim	0.216	0.465	1.130	0.001	2.230	0.004	3.459	0.002	2.608	0.000	1.689	0.120

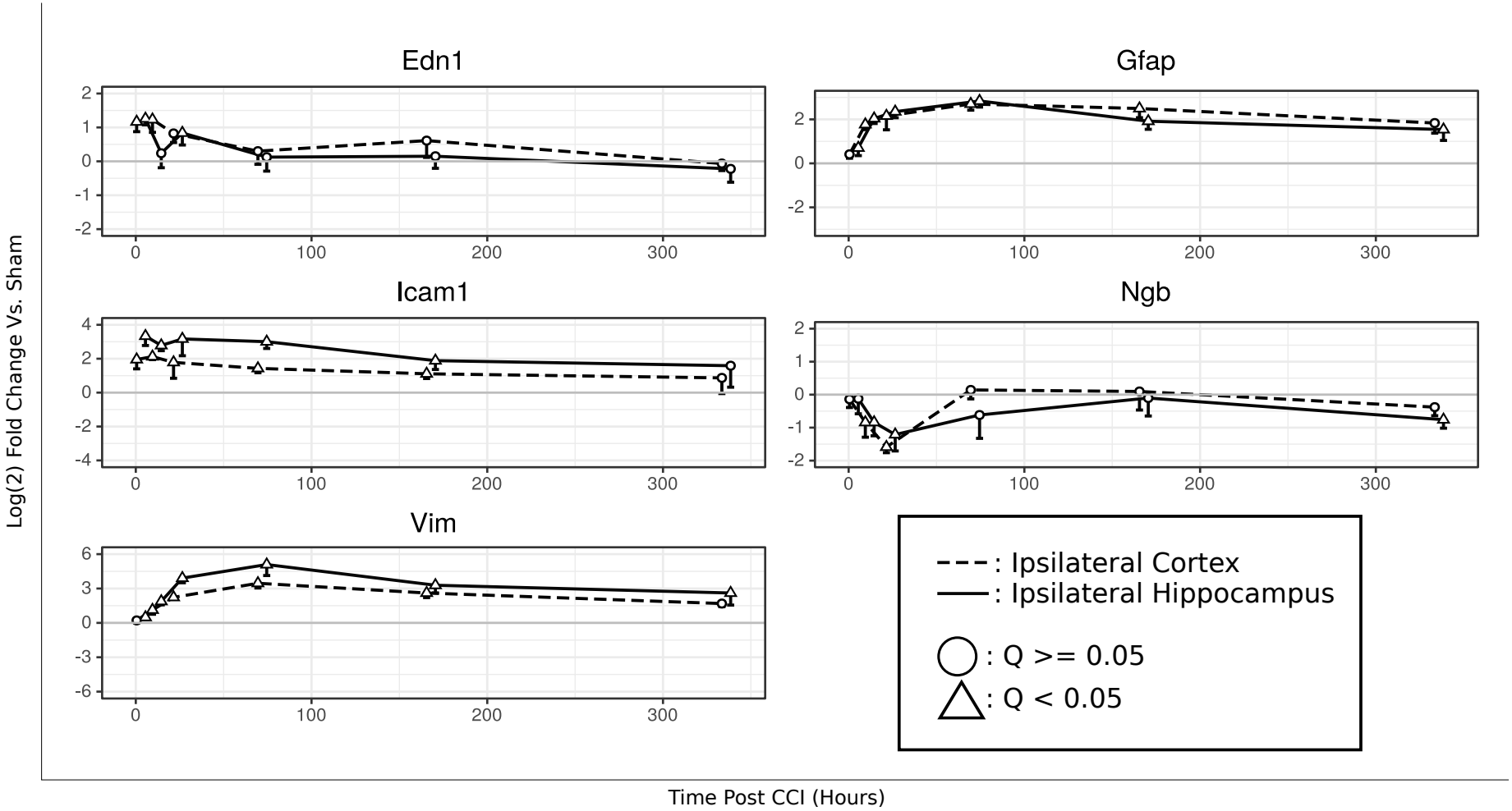

Figure 2.10: Time-course expression of hippocampus and cortex Biomarkers.

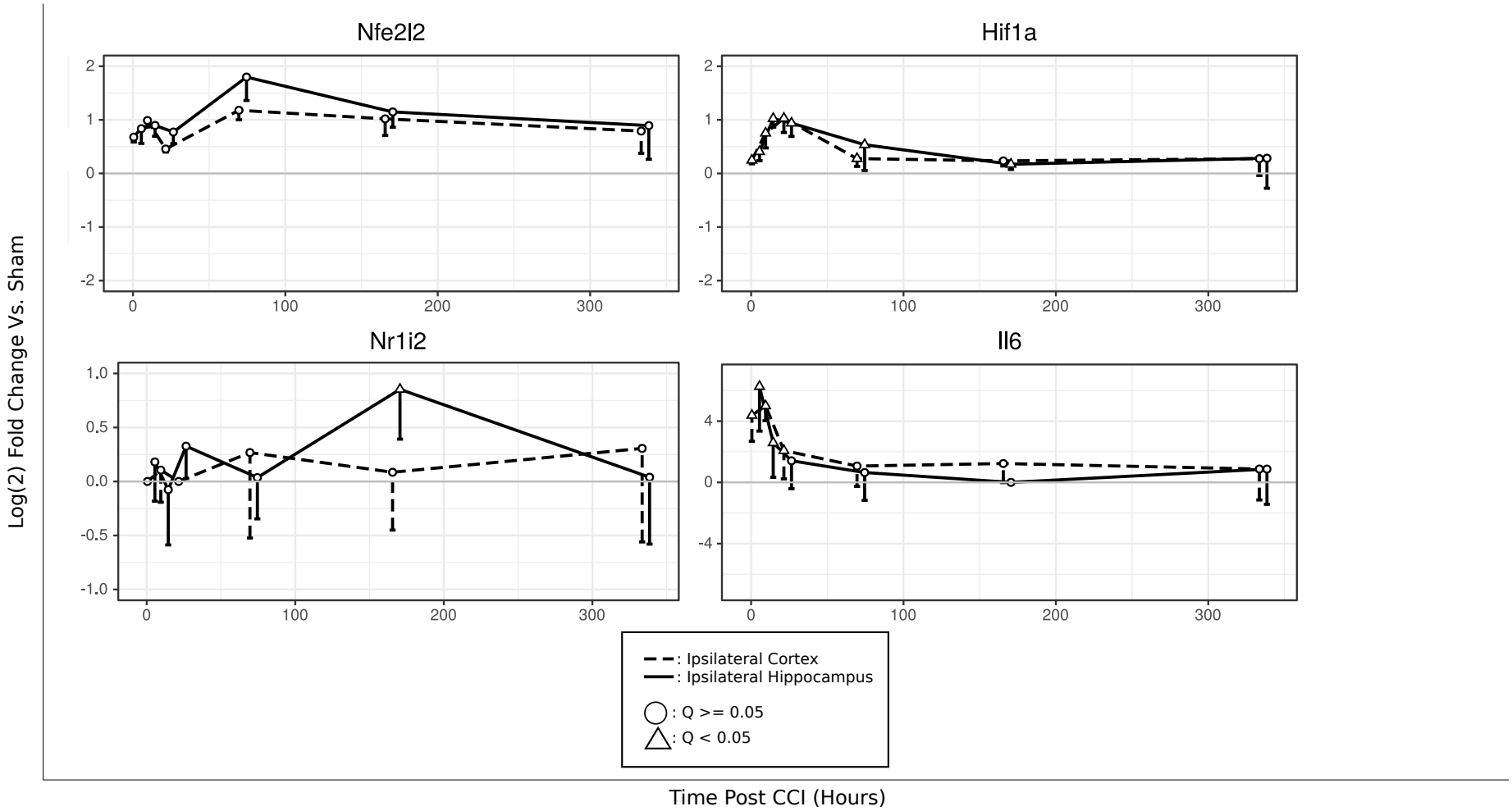

Figure 2.11: Time-course expression of hippocampus and cortex Transcription Factors

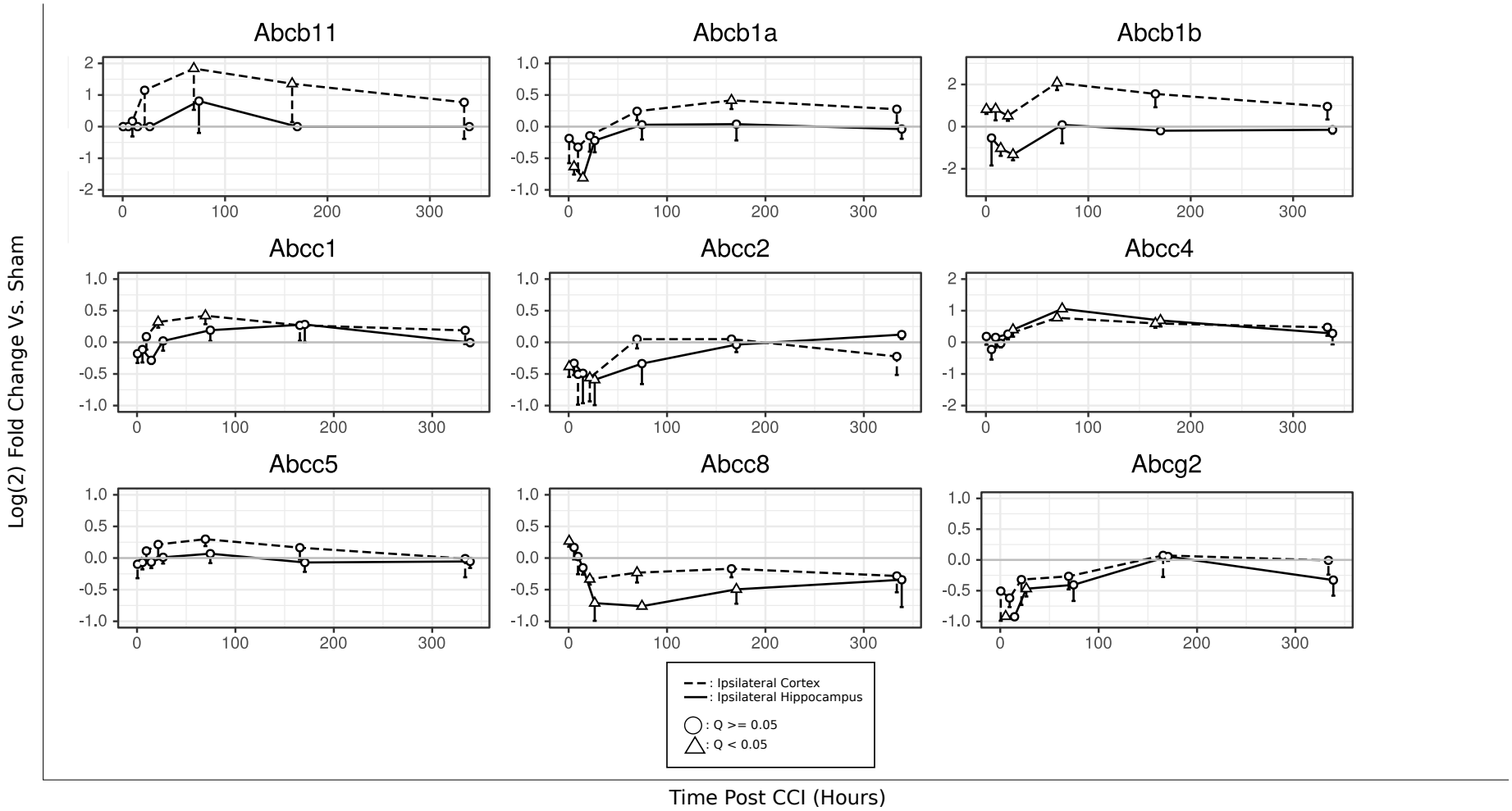

Figure 2.12: Time-course expression of hippocampus and cortex Abc Transporters.

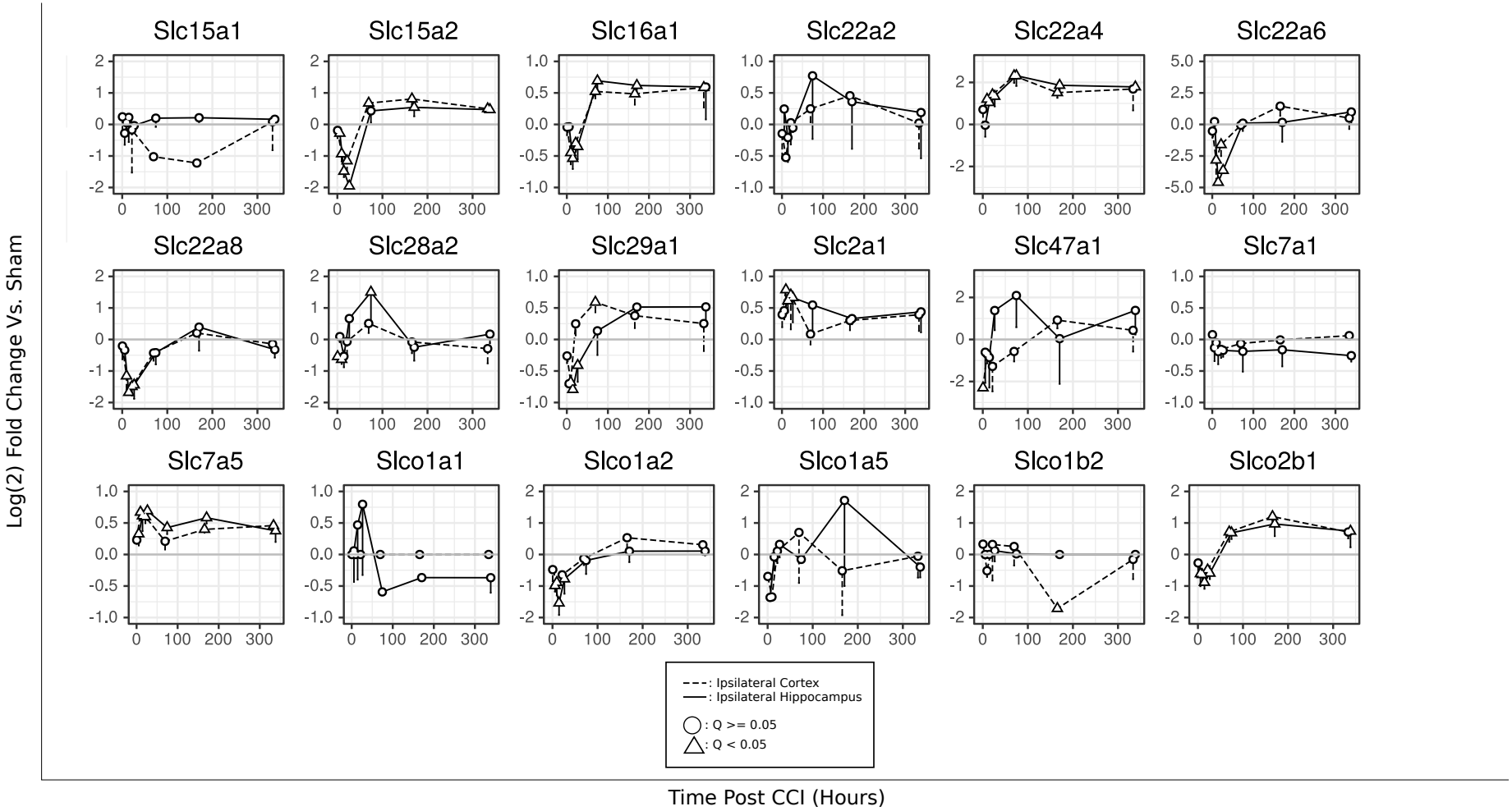

Figure 2.13: Time-course expression of hippocampus and cortex Slc Transporters.

Pathway analysis showed similar mix of enriched pathways in the cortex and hippocampus with a non-zero z score. Patterns showed activations of pathways known to decrease damage to the brain, which were more sustained in the cortex **Figure 2.14**. These were primarily driven by up-regulation of Il-6, Nfe2l2, and Hif1a. The overall pathway for molecular transport in the cortex showed decreased expression early following injury with a switch to activation later **Figure 2.14A**. The hippocampal molecular transporter pathway showed immediate activation, followed by intermediate decrease, and later activation **Figure 2.14B**. A cursory analysis of likely upstream regulators identified the transforming growth factor-beta 1 (max z-score: 0.902) and beta-estradiol (max z score: 0.686) as likely upstream regulators in the cortex and hippocampus.

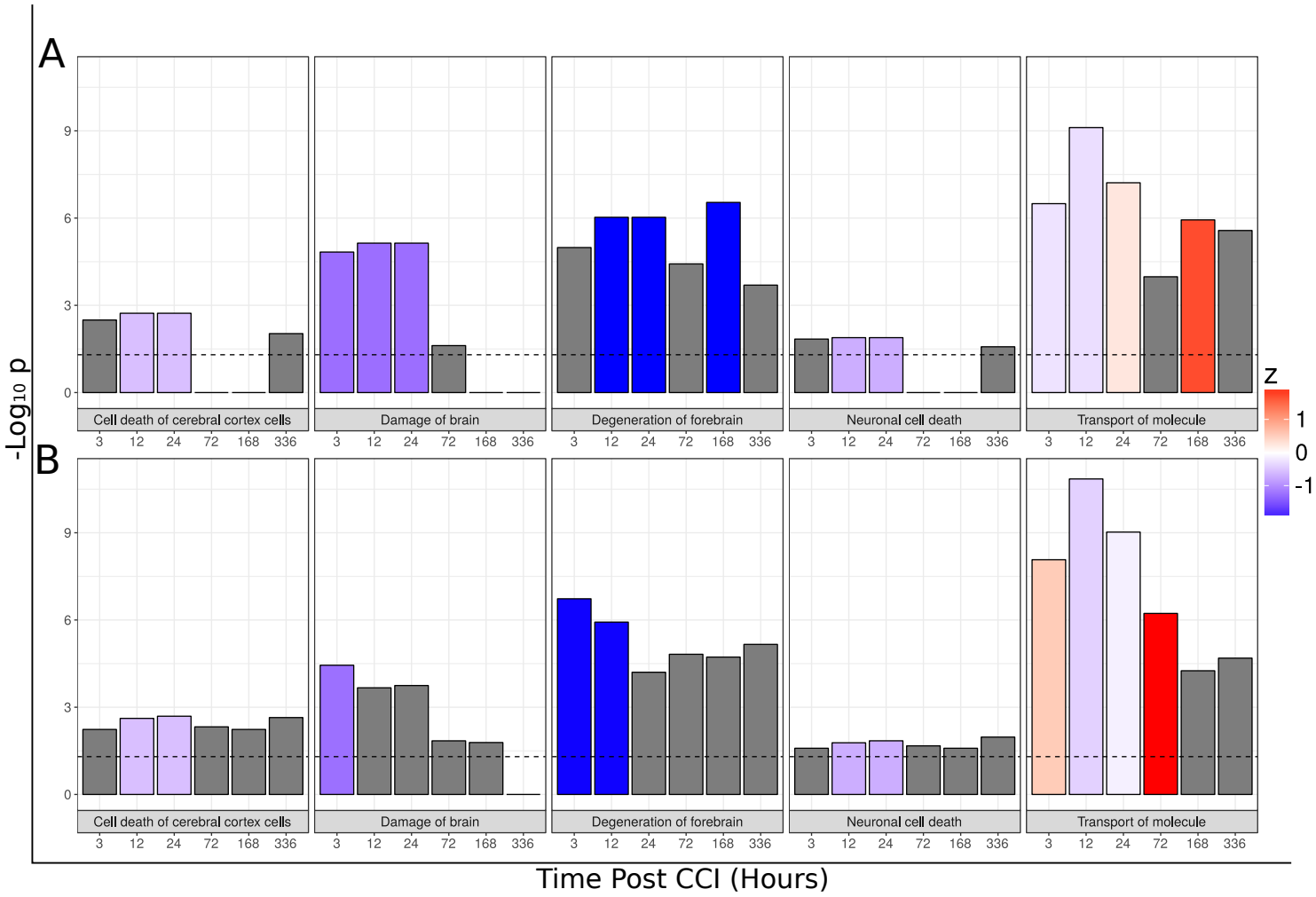

Figure 2.14: Pathway analysis identified several enriched pathways in the ipsilateral hippocampus.

2.3.3.2 Ipsilateral vs. Contralateral

When comparing expression in the ipsilateral cortex vs. contralateral cortex, we found significant changes in 14 genes. Conversely, in the hippocampus we only found significant deviation in two genes. Most of these associations overlapped with the findings in CCI vs. sham, with the exception of Abcb11 in the hippocampus, where we found significantly increased expression over a baseline and CCI level of zero. Overall, these findings suggest that the contralateral side behaves similarly to uninjured brain, supporting the role of the CCI model as a focal injury model with minimal diffuse injury. Findings from this analysis are summarized in **Figure 2.15**.

2.4 DISCUSSION

We investigated baseline transporter expression in pediatric (PND 17, PND 31) and adult rats, and differential expression of transporters in PND 17 rats injured with the CCI model for severe TBI across six time points. Baseline expression of transporters was similar in the cortex and hippocampus, with several gene expression changes associated with development from PND 17 to adulthood. With few exceptions, the majority of ABC and SLC transporters showed early decreases in expression associated with CCI with a gradual return to baseline. Interestingly, we found that neuroglobin was acutely down-regulated following CCI in both cortex and hippocampus. Pathway analysis identified similar patterns of enriched pathways and upstream regulators in hippocampus and cortex, with probable upstream regulation driven by transforming growth factor-beta 1 (Tgfb1) and beta-estradiol.

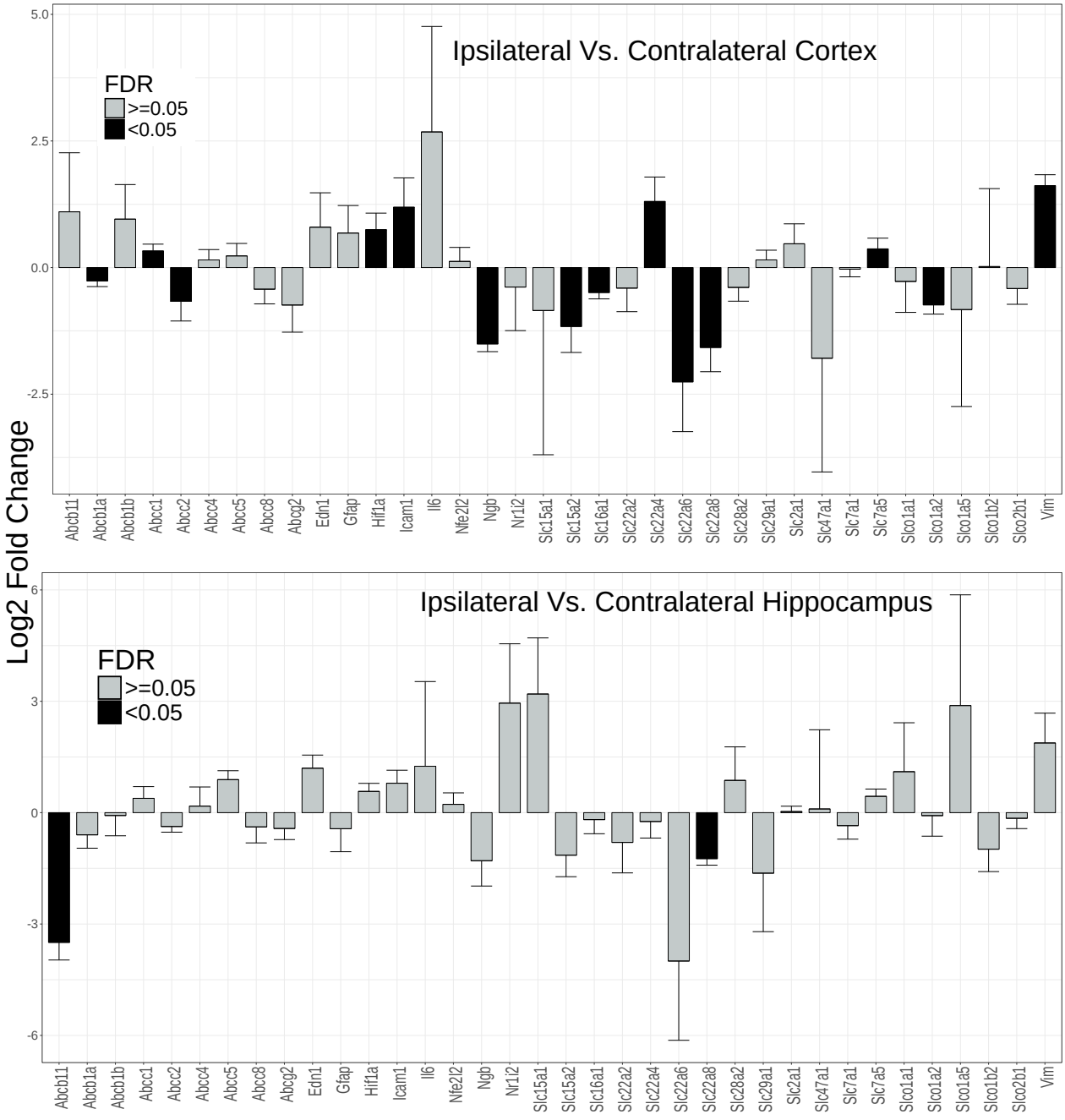

Figure 2.15: Ipsilateral vs. Contralateral Expression at 24 hours in hippocampus (top) and cortex (bottom).

2.4.1 Baseline expression shows diversity with development from PND 17 to adulthood

Transporter expression in PND 17 rats was similar in relative magnitude across genes, but showed significant intra-gene variation through adulthood. This is similar to findings in the gut found by Mooij and colleagues, which show changes associated with development alone.[148] This suggests that, similarly to the approach with gut expression of drug metabolizing genes, brain disposition of xenobiotics should be considered when extrapolating adult doses to pediatrics.[148] This is exemplified by our findings in the Abcc (MRP), Abcb (MDR), and Slco (OATP) transporter families, which showed significant diversity within the hippocampus and cortex.

We found that Slc15a1 (human homologue: SLC15A1) is expressed in the brain at PND 17 and 31, but did not detect expression in adults. This peptide uptake transporter has previously been only associated with intestinal brush border expression, but our findings suggest that it may be expressed in the brain and/or blood-brain barrier in young rats.[149] We found that a member of the same (PEPT) family of transporters, Slc15a2, was expressed at similar levels in all ages of rats. This is primarily expressed within the brain on astrocytes and on endothelial cells on the choroid plexus.[150] Slc15a2 transports small polypeptides and peptide-like drugs (e.g. beta-lactam antibiotics). It's expression in the brain is on the blood-CSF barrier and within the brain parenchyma, and it is expressed on astrocytes in neonates only.[151] Our finding that Slc15a1, which has similar substrates but different affinity and capacity, is expressed in the hippocampus and cortex in pediatrics suggests that pediatric disposition of these substrates in the brain is more variable than previously thought.[149] Additional study will be necessary to replicate and identify the localization of Slc15a1

expression in the pediatric brain.

Collectively, these answer fundamental questions about diversity among different brain tissues and how that changes with development. It also shows the magnitude of expression in the brain with respect to organs (liver and kidney) with generally high levels of transporter expression.

2.4.2 Transporter expression follows a similar pattern in hippocampus and cortex following CCI

Overall, we found that transporter expression shows patterns of early down-regulation followed by delayed increase in transporter expression. This is corroborated by the pathway analysis, which showed a similar pattern of decreased and increased expression. We found that Abcb11, which is normally not present above minimum levels in the brain, was increased with similar timing as a spike in Nfe2l2 at 72 hours post injury.[134] While this spike was transient in the hippocampus and may not be sufficient to lead to protein expression, it was sustained in the cortex following injury.

These changes in expression mirror changes in expression of transcription factors that can impact the expression of metabolic proteins (e.g. enzymes, transporters). Particularly, early inhibitions in expression of transporters mirror changes found in Il-6, which is associated with decreased expression of transporters and cytochrome P-450 enzymes.[152, 128] This acute decrease in expression is likely aimed at protection of tissue from the toxic byproducts associated with injury and tissue repair.[153] Delayed increases in expression follow patterns, most pronounced in Hif1a.

2.4.3 Neuroglobin expression post CCI was divergent from expectation

Our study showed that neuroglobin expression decreased in the hippocampus and cortex following TBI. Neuroglobin is a neuro-protective hemoprotein that binds O_2, NO, and CO in the brain.[154] It can mitigate oxidative stress and hypoxia, and over-expression is neuroprotective.[154, 155] Previous work by Di Pietro and colleagues studied neuroglobin expression following experimental diffuse (mild and severe) TBI in adult rats. They found increased expression of neuroglobin immediately following injury in both the severe and mild TBI models.[154] This suggests divergence with the brain's response to our focal injury model (CCI) versus the response following a diffuse injury model. This contrasts with the other biomarkers evaluated on this panel, which followed expected patterns of expression changes.

2.4.4 Similar pathway enrichment found in cortex and hippocampus

Pathway analysis identified similar patterns of enrichment in hippocampus and cortex. Upstream regulator analysis identified Tgfb1 and beta-estradiol as the most likely expression regulators in pediatric experimental severe TBI. This is corroborated by work by Kobori and colleagues, who found that Tgfb1 expression was increased within two hours of CCI in mice.[156] Tgfb1 is associated with decreased inducible nitric oxide synthase (iNOS) related to activated microglia and macrophages.[157] Broader pathway analysis carried out by Samal, et al. identified Tgfb1 as part of the limited set of ten first messengers that explains approximately 97% of downstream gene expression changes at 24 hours post mild CCI.[158] Beta-estradiol is generally regarded as a neuro-protective hormone.[159, 160]. It is also associated with a rapid decrease in Abcg2 expression and function[161] and increased oxidative metabolism

of dopamine[162].

2.4.5 Abcg2

While this particular project focused on the overall changes in transporter mRNA expression, we are particularly interested in how Abcg2 is impacted by injury. Understanding how Abcg2 expression changes with animal development in addition to changes following severe TBI will better help to understand findings in studies in the following chapters.

We found that Abcg2 was consistently expressed at high levels in the cortex and hippocampus at all time points tested, whereas expression in the choroid plexus showed relatively low expression. This suggests that at baseline, the contribution of Abcg2 to substrate efflux on the choroid plexus is lower than the brain/BBB. Warren and colleagues studies the comparative expression of Abc/ABC transporters in rodent and human brains. They found that Abcg2 was more highly expressed relative to Slc2a1 in rodents than in humans.[134]

Abcg2 was remarkably consistent in the cortex and hippocampus following experimental TBI. It showed significant decreased in expression at the three hour time point following injury, but was back to baseline by the 12 hour time point. This suggests that Abcg2 may not as sensitive as other Abc transporter to inflammation.

2.4.6 Limitations

Our pathway analysis is based on a relatively small set of genes that are centered around transporters, thus findings are likely to be biased to those genes. Our model of severe TBI (CCI) is a well studied focal model, but may not translate to a diffuse

injury model. To minimize time from dissection to freezing, we rinsed tissue with saline rather than perfusing the rats. This raises the possibility of contamination of mRNA found in blood and can raise the risk for noise in results. For this analysis, we did not validate changes in mRNA expression with protein expression analysis. While the mRNA data provides insight into the biological mechanisms, it does not always translate to changes in protein levels.

2.5 CONCLUSION

We investigated transporter mRNA expression at baseline and in experimental TBI at acute and long term time points following injury in pediatric rats using the innovative expression technology, Nanostring, that provides absolute transcript counts in multiple brain tissues. The findings are an important step toward stronger understanding of secondary injury following TBI in children, and draws interesting contrast to previous investigations into expression changes following TBI. Baseline expression changes associated with development from pediatric to adulthood suggest a dynamic biochemical environment that may have implications in drug development and dosing. These baseline expression levels also differ across brain tissues suggesting that the disposition of xenobiotics and endogenous substrates is dynamic across brain regions and barriers. Experimental TBI in pediatric rats shows patterns of acutely decreased transporter expression in the cortex and hippocampus, with some divergent increases in expression at various time points. This is associated with acute increases in the inflammatory cytokine, Il-6, and later increases in the transcription factor, Hif1a, suggesting a dynamic interplay between factors that inhibit and induce expression. Changes in

expression from the acute to chronic phase post TBI may support the use of dynamic treatment protocols that call for variable dosing with time post injury.

3.0 ASSOCIATION OF ABCG2 GENETICS AND CLINICAL OUTCOMES FROM SEVERE TRAUMATIC BRAIN INJURY

[Adams SM, et al. *J. Neurotrauma* 2018]

Excerpts reprinted with permission from the Journal of Neurotrauma[163]

See Appendix 3 Figure C2

3.1 INTRODUCTION

3.1.1 ABCG2 as a Mediator of Brain Recovery

ABCG2 is a transporter expressed throughout the brain and is of particular interest because it is highly expressed at all points in human development and it may be responsible for efflux of toxic waste products from the brain (e.g. protoporphyrin IX), xenobiotics (e.g. rosuvastatin, glyburide), and the endogenous antioxidant, uric acid (UA).[164, 165] With this information, three arguments can be made for the potential role of ABCG2 in TBI: 1) ABCG2 function is critical for removal of toxic buildup of protoporphyrin IX and dysfunction could mean increased brain toxicity; 2) ABCG2 is a mediator of the disposition of drugs (xenobiotics) used to treat TBI, and variation in ABCG2 function could help precision dosing; and 3) ABCG2 dysfunction promotes higher levels of the antioxidant, UA, and better brain recovery from oxidative stress.

3.1.1.1 Protoporphyrin IX

Protoporphyrin IX (PPIX) is a required intermediary in the heme bio-synthesis pathway and is an ABCG2 substrate.[166] Excess PPIX can cause damage through phototoxicity, and liver accumulation of PPIX can cause hepatic injury through blockade of bile ducts.[166] In the mitochondria, ferrochelatase catalyzes the chelation of iron by PPIX to produce heme.[166] PPIX may be effluxed from the mitochondria to the cytosol by ABCG2, and subsequently to the extracellular space by ABCG2.[166] ABCG2 function is critical to the porphyrin homeostasis in embryonic stem cells, and ABCG2 dysfunction can lead to excess oxidative stress due to accumulation of protoporphyrin IX.[167]

The role of PPIX in TBI is not well established, various forms have shown both toxic and protective roles in the brain. Over-accumulation of heme byproducts may

result from hemoglobin breakdown following brain hemorrhage/contusion, and may contribute to secondary injury from TBI.[168] One such byproduct, PPIX chelated with iron (hemin), may have a neuroprotective effect by inducing expression of heme oxygenase-1 (HO-1), though this effect is only found at specific concentrations and hemin can be toxic in amounts generated from heme breakdown associated with hemorrhage.[169] Conversely, exogenously administered PPIX complexed with zinc (Zn-PPIX) is an inhibitor of HO-1 and has been found to attenuate edema and BBB disruption in experimental closed head injury.[170] HO-1 is upregulated following brain hemorrhage and TBI, and the neuroprotective effect of Zn-PPIX may stem from its ability to attenuate this upregulation.[171]

3.1.1.2 Xenobiotics Relevant to TBI

Rosuvastatin (and possibly other statins) may have an anti-inflammatory effect post TBI, and has been investigated in some small clinical trials with modest success.[3, 172] Rosuvastatin is a substrate for ABCG2 and its pharmacokinetics and efficacy may be impacted by ABCG2 function. Rosuvastatin efficacy as a cholesterol lowering medication has been found to be associated with the *ABCG2* c.421C>A variation, where individuals with the AA genotype have approximately 7% greater decrease in LDL-C.[173] Zhang and colleagues showed that *ABCG2* c.421C>A CA and AA genotypes are associated with approximately 80% higher AUC (p=0.018) and 90% higher C_{MAX} (p=0.048).[174]

Glyburide has been investigated as potential therapeutics for TBI and has shown strong promise in clinical trials for TBI.[3] The benefit of glyburide is thought to be related to inhibition of the ABCC8-TRPM4 channel as a strategy to mitigate cerebral edema.[31, 104] Pollex and colleagues investigated the impact of HEK-293 cells trans-

fected with the *ABCG2* c.421C>A variant on intracellular glyburide accumulation (an indirect measure of ABCG2 affinity). They found that the "A" allele at c.421C>A was associated with significantly higher accumulation, suggesting decreased affinity for glyburide.[175] While this has not been evaluated clinically, it is possible that *ABCG2* c.421C>A may have utility as a PGx predictor of glyburide pharmacokinetics. Given that in TBI the dose of glyburide is generally lower than when used for type 2 diabetes, and that ABCG2 may limit its entry into the brain - it is possible the patients with variant ABCG2 may require lower doses of glyburide to achieve adequate brain concentrations.

3.1.1.3 Uric Acid

Uric acid is the highest abundance antioxidant present in the blood and is a significant part of the antioxidant reserve in the brain, in addition to ascorbic acid, glutathione, and several other compounds.[108] TBI is associated with massive generation of reactive oxygen species (ROS), and strategies to increase the oxidative reserve post-TBI have been popular in TBI drug development.[31, 108] *ABCG2* c.421C>A is strongly associated with risk for hyperuricemia and gout, along with several other less common variants in the *ABCG2* gene.[176] This association is due to decreased ABCG2 protein expression conferring decreased renal and extra-renal (e.g. gut) clearance of UA.[177, 178] Given the high level of expression of ABCG2 on the blood-brain barrier, it is possible that individuals who carry an "A" allele at *ABCG2* c.421C>A have higher brain concentrations of UA at baseline, thus a more robust antioxidant reserve. Conversely, the "A" allele at *ABCG2* c.34G>A may confer lower brain concentrations of UA and a weaker antioxidant reserve.

3.1.2 Objective and Hypothesis

We were interested in studying the role of *ABCG2* increased (c.34G>A) and decreased (c.421C>A) function with regards to clinical TBI outcomes following the UA hypothesis. Our objective was to determine the potential association of *ABCG2* c.421C>A and c.34G>A with outcomes following severe TBI in multiple cohorts. We hypothesized that genetic variation causing decreased ABCG2 function/expression would be associated with improved clinical outcomes (i.e. improved GOS score) following TBI.

3.2 METHODS

3.2.1 Population

This was a retrospective cohort study based on data collected from the University of Pittsburgh Brain Trauma Research Center (BTRC) . The BTRC contains injury data, outcomes data, and biologic samples from patients suffering from TBI who consent to research. Subjects were identified from the BTRC following approval by local Institutional Review Board. Informed consent was obtained from the subjects' surrogates prior to data collection. Criteria for inclusion were: age 16 and older, Glasgow Coma Scale (GCS) score of 8 or less in the absence of medications known to affect GCS, and external ventricular drain (EVD) placement. To diminish the risk for population stratification skewing results, only Caucasian subjects were included in the final analysis. Patients with penetrating trauma and those with previous neurologic impairment were excluded. Two independent cohorts were identified to provide a cohort for discovery and replication. Cohorts were built by date of injury (separated by approximately four years) from the same clinical site.

3.2.1.1 Outcome Measurement

Glasgow Outcome Scale (GOS) scores were collected by qualified neuro-psychologists 3, 6, 12, and 24 months post injury. The GOS ranges from 1 to 5, where a score of 1 corresponds to deceased, 2 corresponds to a patient in a persistent vegetative state, 3 corresponds to a patient with severe disability, 4 corresponds to a patient with minor disability, and 5 corresponds to little or no disability. To control for injury characteristics based on pathoanatomical data, computed tomography imaging data was reviewed for hemorrhage types associated with the injury. Patients were coded based on the presence or absence of one or more of the following: epidural hematoma (EDH), subdural hematoma (SDH), subarachnoid hemorrhage (SAH), intraparenchymal hemorrhage (IPH), intraventricular hemorrhage (IVH), and/or evidence of diffuse axonal injury (DAI).

3.2.2 Genotyping

DNA was isolated from CSF samples taken from the EVD or from blood samples using a commercially available kit (Qiaamp kit, Qiagen, Chatsworth, CA, USA). Genotyping for *ABCG2* c.421C>A and c.34G>A was performed with a TaqMan™allelic discrimination assay for the discovery cohort (Applied Biosystems, Foster City, CA, USA), and a mix of TaqMan™+ Illumina Human Core Exome v1.2 (Illumina, San Diego, CA, USA) for the replication cohort. Genotype data quality was addressed by using technical replicates, blinded double calls of raw data, assessment for significant deviation from Hardy-Weinberg equilibrium. Allele frequencies were compared with published frequencies for the non-Finnish European population from the Broad Institutes's gnomAD Browser.

3.2.3 Statistical Analysis

All statistical analyses were carried out using R v3.4.3 (R Development Core Team, Vienna, Austria). Demographic data for the discovery and replication cohorts were compared using the t-test, Chi-Square test, or Fisher's exact test. All genetic association studies were carried out using a dominant variant allele model (i.e. Phenotype magnitude in CC < CA/AA). Subjects with missing hemorrhage-type data were coded with a dummy variable to account for the missing data while avoiding list wise exclusion.

3.2.3.1 Mixed Ordinal Regression Model

To determine the association of *ABCG2* c.421C>A and c.34G>A with outcomes, GOS score was treated as an ordinal variable and compared with mixed effect multiple ordinal regression using the package Ordinal for R. Covariates in the model included age, sex, GCS score, post-injury imaging derived hemorrhage types, and time-post injury. All covariates were tested individually and for two-way interactions, and were forced into the model due to their previously determined associations with TBI severity and outcomes.[179, 180] Time post injury was also controlled for to delineate the multiple measurements for each level of the random variable (subject ID). Skewed continuous variables were transformed by taking the natural log to improve model fit. Significance for random effects were determined with ANOVA of a mixed model and a standard model excluding the random effect variable. Models were fit using adaptive Gauss-Hermite approximation with variable Gaussian quadrature points to attain the most stable model. The following code provides an example for how the command is called.

```
require(ordinal)
```

```
ordinal.model <- clmm(data, y ~ x1 + x2 + ... + xn + (1|ID), Hess = TRUE,
    nAGQ = q, doFit = T)
```

Results are presented as model parameters and odds ratios where appropriate, which correspond to the odds of being in any higher GOS level. Depending on the presence of interacting terms, odds ratios will be calculated conditionally upon interacting terms using

3.2.3.2 Testing for Proportionality of Odds

Ordinal logistic regression is an extension of logistic regression that allows for a multi-level dependent variable (ordinal). This model assumes proportional odds, which states that the odds of being in a higher level are the same. For example, with the GOS score, proportional odds being met states that the odds of being GOS 1 vs. GOS 2, 3, 4, or 5 are the same as being GOS 1,2 vs. GOS 3, 4, or 5, and so on. While there are multiple methods to test this assumption in simple models, at the time of this analysis there are no well-established methods for assessing proportionality of odds adherence in mixed ordinal regression models. Bell and Dexter suggested splitting ordinal variables in all possible cut-points and comparing each model for similarity (i.e. the slopes will be the same in each model with differing intercepts.)[181] To apply this method to our data, we dichotomized the GOS along four possible cut-points [1|2-5, 1-2|3-5, 1-3|4-5, 1-4|5], then controlled for age, sex, time, post-injury imaging determined hemorrhage types, and GCS with random effects of subjects with mixed-effects binomial logistic regression using the LME4 package for R. Confidence intervals (95%) of the coefficients were estimated using:

$$f(x, y) = x \pm 1.96 * y$$

were compared for all coefficient estimates for each break point. The model was controlled for time post injury, age, sex, time post-injury, imaging determined hemorrhage types, and GCS. If the main effects (genotype and any interaction terms) 95% confidence intervals shared overlap, it was assumed that proportionality of odds was met.

3.2.3.3 Univariate Assessment of Dichotomized Outcomes

To obtain a simple estimate of odds of having a favorable (GOS > 3) vs. unfavorable (GOS < 4) outcome, we measured the proportion of subjects in each group split by genotype and any relevant interactions found in the mixed effect model. This was compared with the chi-square test at each time point post injury.

3.3 RESULTS

3.3.1 Demographics and Population Characteristics

The discovery and replication cohorts contained 270 and 166 subjects, respectively. **Table 3.1** shows the demographic information for each cohort.

Table 3.1: Cohort Demographics

Factor	Discovery Cohort	Replication Cohort	p value
Age Median (IQR*)	33 (23-47)	36.5 (24-54)	0.03
Female (%)	21.1%	22.3%	0.86
GCS** Median (IQR*)	6 (4-7)	7 (5-7)	0.22
Hemorrhage Type			
None	2 (0.7)	2 (1.2)	1
Epidural Hematoma	29 (10.7)	17 (10.2)	0.99
Subdural Hematoma	134 (49.6)	49 (29.5)	<0.001
Subarachnoid Hemorrhage	132 (48.9)	43 (25.9)	<0.001
Intraparenchymal Hemorrhage	65 (24.1)	39 (23.5)	0.98
Intraventricular Hemorrhage	54 (20.0)	7 (4.2)	<0.001
Diffuse Axonal Injury	58 (21.5)	21 (12.7)	0.03
No Imaging Data	46 (17.0)	24 (14.5)	0.56
ABCG2 c.421C>A (CC) N (%)	227 (84.1)	128 (77.1)	
ABCG2 c.421C>A (CA/AA) N (%)	43 (15.9)	38 (22.9)	0.09
ABCG2 c.34G>A (GG) N (%)	238 (93.0)	115 (92.7)	
ABCG2 c.34G>A (GA/AA) N (%)	18 (7.0)	9 (7.3)	1

*IQR: Interquartile Range

Notably, subjects in the replication cohort were significantly older than the discovery cohort. The frequency of the "A" (variant) allele in c.421C>A was higher in the replication cohort than the discovery cohort, though this did not reach statistical significance (p=0.09). We also found that the distribution of hemorrhage types were significantly different among the two populations, suggesting that the granular injury details differed within the populations.

ABCG2 c.421C>A and c.34G>A adhered to Hardy-Weinberg equilibrium in both cohorts separately and combined ($p > 0.05$). For c.421C>A, calculated minor allele frequencies (MAFs) were 7.96%, 12.35%, and 9.63% in the discovery, replication, and combined cohorts, respectively. These did not significantly deviate from MAF for *ABCG2* c.421C>A in the non-Finnish European population based on the gnomAD

Browser (discovery p=0.08, replication p=0.27, combined p=0.52). For c.34G>A, calculated minor allele frequencies (MAFs) were 3.5%, 3.6%, and 3.6% in the discovery, replication, and combined cohorts, respectively. These also did not significantly deviate from the MAF for *ABCG2* c.34G>A in the non-Finnish European population based on the gnomAD Browser (discovery p=1, replication p=1, combined p=1).

3.3.2 Association of Genotype with Outcomes

3.3.2.1 Model Building

Preliminary analyses showed a significant association of c.421C>A with clinical outcomes, but not with c.34G>A. Final model building proceeded with c.421C>A. The final mixed effects ordinal regression model was built with covariates age, sex, time post-injury, imaging determined hemorrhage types, and GCS forced in the model to control for injury severity and patient characteristics that might impact recovery independently.

3.3.2.2 Proportionality of Odds

The assumption of proportionality of odds was tested and found to be met in nearly all cases. We found that for the time post injury variable and the X intercept had some breakpoints where the 95% confidence interval lacked overlap. We proceeded with the model given that the main effects (genotype) and all others met the prespecified assumption. The estimated confidence intervals are shown in **Table 3.2**. Of note, there is missing overlap in the time post injury (Time) and the X intercept in some measures, but all other parameters were found to overlap. Despite these minor deviations, the assumption of proportional odds was accepted. The R code used to calculate these is in appendix B.

Table 3.2: The proportional odds assumption was tested by comparing confidence intervals of each paramater at each posible breakpoint of GOS score. Breakpoints for each model are specified with "|", where scores to the left are coded as 0, and to the right as 1. Lower refers to the lower bound of the 95% confidence interval estimate for the coefficient, and upper to the upper bound.

Parameter	1|2345		12|345		123|45		1234|5	
	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
X Intercept	2.20	23.51	-1.88	16.45	-4.84	5.35	-6.58	1.90
rs2231142	-8.66	42.27	-4.74	38.27	-0.21	20.61	0.47	16.81
log Age	-7.27	-1.84	-5.89	-1.22	-3.72	-1.17	-2.79	-0.69
Sex	-2.96	1.94	-2.31	1.93	-0.37	2.08	-0.74	1.26
Time	-0.18	-0.06	-0.05	0.04	0.09	0.15	0.10	0.16
None	-8657.34	8694.92	-5165.36	5203.01	-1.60	8.70	-0.93	6.30
EDH	-2.33	5.03	-2.35	3.79	-0.28	2.83	-0.13	2.35
SDH	-3.03	1.63	-2.79	1.27	-1.54	0.67	-0.83	1.01
SAH	-2.79	1.91	-2.43	1.66	-1.05	1.18	-1.15	0.71
IPH	-1.81	3.41	-1.53	3.06	-0.45	2.04	-0.19	1.85
IVH	-2.29	4.28	-2.19	3.49	-1.55	1.46	-1.36	1.11
DAI	-2.71	3.51	-2.77	2.55	-0.66	2.13	-0.10	2.12
no data	-4.05	3.01	-4.16	2.00	-1.55	1.79	-0.62	2.10
GCS	0.54	1.94	0.65	1.87	0.43	1.12	0.23	0.81
rs2231142*log(Age)	-11.57	2.12	-10.51	1.14	-5.73	0.21	-4.73	0.09

3.3.2.3 Model Results

The final model for the discovery cohort and replication cohorts are summarized in **Table 3.3**. We found a significant association with the "A" allele at c.421C>A in both cohorts [p = 0.01 (discovery) and p = 0.02 (replication)]. Additionally, we found a significant interaction between the presence of the "A" allele at c.421C>A and age [p = 0.03 (discovery) and p = 0.01 (replication)].

Table 3.3: Mixed Effect Ordinal Regression Models for Discovery and Replication Cohorts

Factor	Discovery Cohort		Replication Cohort	
	Coefficient (95%CI)	p	Coefficient (95%CI)	p
ABCG2 c.421C>A	22.03 (4.53; 39.53)	0.01	25.94 (4.47; 47.42)	0.02
ln(Age)	-6.69 (-9.01; -4.37)	<0.001	-4.37 (-7.21; -1.53)	0.003
ABCG2 c.421C>A * ln(Age)	-5.58 (-10.56; -0.60)	0.03	-8.03 (-14.18; -1.88)	0.01
GCS** Score	2.18 (1.55; 2.82)	<0.001	1.47 (0.68; 2.27)	<0.001
Hemorrhage Type				
None	10.14 (0.95; 19.34)	0.03	2.19 (-7.05; 11.43)	0.64
Epidural Hematoma	-0.37 (-2.99; 2.24)	0.78	5.40 (1.54; 9.26)	0.01
Subdural Hematoma	-0.56 (-2.46; 1.35)	0.57	-1.99 (-4.78; 0.79)	0.16
Subarachnoid Hemorrhage	-1.59 (-3.45; 0.28)	0.1	0.85 (-1.76; 3.47)	0.52
Intraparenchymal Hemorrhage	1.90 (-0.18; 3.98)	0.07	1.25 (-1.72; 4.22)	0.41
Intraventricular Hemorrhage	0.52 (-1.69; 2.72)	0.65	0.19 (-5.11; 5.50)	0.94
Diffuse Axonal Injury	1.48 (-0.79; 3.75)	0.2	1.04 (-2.29; 4.37)	0.54
No Imaging Data	-2.55 (-5.57; 0.48)	0.1	2.32 (-1.42; 6.05)	0.22
Sex (M v. F)	1.01 (-0.99; 3.01)	0.32	-0.52 (-3.07; 2.02)	0.68
Months Post Injury	0.12 (0.09; 0.14)	<0.001	0.09 (0.05; 0.13)	<0.001
Subject (Random Effect)	N/A	<0.001	N/A	<0.001

Finally, both cohorts were combined into a final group, and assessed with the same model covariates. The coefficient for the conditional (genotype) effects were positive (23.56, $p < 0.001$), and the coefficient of the interaction was negative (-6.69, $p < 0.001$), suggesting that the effect of genotype is mitigated by increasing age. The final full model is summarized in **Table 3.4**. The odds of a favorable outcome were much higher in younger subjects with at least one variant allele at *ABCG2* c.421C>A, and this effect is adjusted with age.

Table 3.4: Mixed Effect Ordinal Regression for Combined Cohort

Factor	Coefficient	SE(95%CI)	p
ABCG2 c.421C>A	23.56	6.68 (10.46; 36.66)	<0.001
ln(Age)	-5.93	0.92 (-7.73; -4.12)	<0.001
ABCG2 c.421C>A * Age	-6.69	1.89 (-10.39; 2.99)	<0.001
GCS** Score	1.89	0.25 (1.40; 2.38)	<0.001
Hemorrhage Type			
None	5.54	3.35 (-1.03; 12.12)	0.1
Epidural Hematoma	2.12	1.09 (-0.01; 4.25)	0.05
Subdural Hematoma	-0.9	0.76 (-2.39; 0.59)	0.24
Subarachnoid Hemorrhage	-0.45	0.75 (-1.92; 1.02)	0.55
Intraparenchymal Hemorrhage	1.45	0.85 (-0.22; 3.12)	0.09
Intraventricular Hemorrhage	0.47	1.02 (-1.52; 2.46)	0.64
Diffuse Axonal Injury	1.16	0.97 (-0.74; 3.05)	0.23
No Imaging Data	-0.31	1.15 (-2.56; 1.95)	0.79
Sex (M v. F)	0.29	0.81 (-1.29; 1.87)	0.72
Months Post Injury	0.11	0.01 (0.09; 0.13)	<0.001
Subject (Random Effect)	N/A	N/A	<0.001

The age at which the effect of age completely mitigated the genotype effect was calculated with the following:

$$age = \exp(\beta_{genotype} / -\beta_{genotype*age})$$

where β refers to coefficients from the combined cohort model defined by the subscript. Based on the combined model, this suggests that the age of inflextion is approximately 34 years, after which having at least one variant allele at *ABCG2* c.421C>A is predictive of a unfavorable outcome compared to those with non-variant alleles. Note that this does not necessarily mean that the association is actually flipped, but just that the effect of genotype above or below 34 drives the findings.

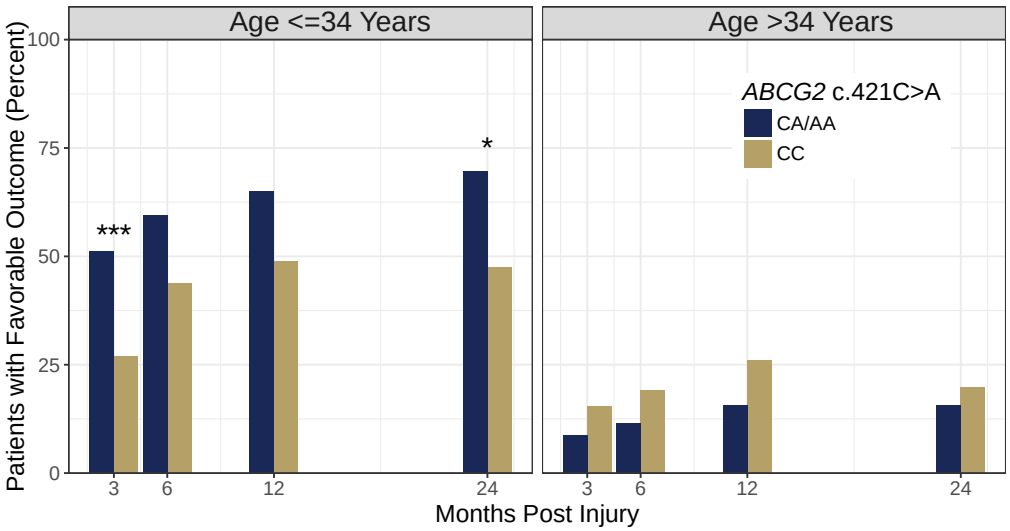

Figure 3.1: When split at the age of inflection (34 years), dichotomized GOS scores show that individuals under 34 had significant benefit from the "A" allele at *ABCG2* c.421C>A, while subjects over 34 did not have benefit or harm.
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3.3.3 Age Based Assessment of Dichotomized GOS Score

Figure 3.1 shows the proportion of subjects with favorable (GOS ≥ 4) outcomes broken up by age >34 and genotype. To determine the odds ratio associated with genotype conditional upon subject age, odds ratios for heterozygote and homozygote vs. wild type were plotted with increasing age bound by the interquartile range **Figure 3.2**.

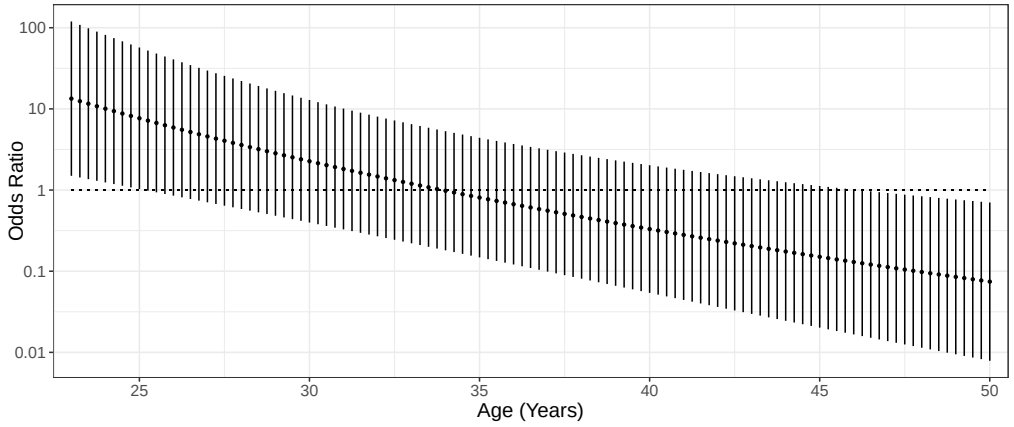

Figure 3.2: Model based odds ratios show a rapid change in benefit from the "A" allele at *ABCG2* c.421C>A with age. This allows visualization of the model interaction.
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3.4 DISCUSSION

In this study we sought to determine the association of *ABCG2* c.421C>A and c.34G>A with outcomes following severe TBI by utilizing a robust discovery-replication method. This allowed for replication in an independent cohort and strengthens the findings. We found similar population characteristics in the discovery and replication cohorts, with notable differences among age and distribution of imaging derived injury severity (i.e. hemorrhage, presence of DAI). This reflects the expected heterogeneity of the TBI population, and the inclusion of these as covariates should provide adequate correction for associated differences. Different distributions among covariates in replicate populations also provides an opportunity to explore the association in a more clinicaly representative population. Preliminary analyses through model building found

that c.34G>A was not associated with clinical outcomes, possibly due to insufficient power based on a lower MAF. We found that subjects with at least one "A" allele at c.421C>A had improved odds of having a higher GOS score, and that the beneficial effect was mitigated by increasing age to a point of inflection at approximately 34 years old. Based on the observed data versus the model, it appears more likely that the genotype effect is present in younger individuals, and is not an important factor in the older population, rather than a reversal of association in subjects over 34 years old.

3.4.1 *ABCG2* c.421C>A Phenotype

The molecular phenotype of *ABCG2* c.421C>A is decreased ABCG2 protein expression due to improper folding, ubiquitination, and proteasome mediated degradation.[182] The consequence of carrying a variant allele at *ABCG2* c.421C>A is decreased expression to half that of wild type ABCG2.[182] Variant alleles are associated with higher risk for gout due to decreased renal and biliary efflux of the ABCG2 substrate, UA, raising the risk for hyperuricemia.[178, 177]

3.4.2 *ABCG2* c.421C>A Association with Other Neuro-Pathologies

Matsuo and colleagues investigated the role of *ABCG2* c.421C>A in the onset of Parkinson's disease under the hypothesis that increased UA in the brain would provide neuro-protection. They found that patients with at least one "A" allele developed Parkinson's disease 1.6 years later than those without a variant allele ($p = 0.025$).[138] This finding suggests that decreased expression and/or function of ABCG2 on the BBB might confer neuro-protection, which might direct new treatment options

for TBI.

3.4.3 Potential Mechanisms

A potential explanation for our findings might be that people who have at least one variant allele at *ABCG2* c.421C>A have decreased efflux of UA from the brain, and thus higher brain concentrations of UA. Increased concentrations may promote brain recovery after TBI. Uric acid elevation therapy has been proposed as a potential treatment option for multiple neurologic pathologies due to its neuro-protective effects. Clinical trials have already investigated the use of exogenous UA as a treatment for ischemic stroke and have provided evidence that UA therapy might prevent early ischemic worsening compared to placebo (p=0.01).[183]

This is supported by UA's endogenous antioxidant activity in the brain and its role in mitigating synaptic glutamate levels via up-regulation of the glial glutamate transporters: SLC1A3 (EAAT-1) and SLC1A2 (EAAT-2). Du and colleagues also found that inhibition of these transporters in an in vitro model for spinal cord injury promoted apoptosis.[184] Thus, combining the antioxidant roles of UA with its anti-glutamate effects strongly promotes the ability of UA, and thus ABCG2 in protecting the brain following injury. Counter to this theory, Maetzler and colleagues evaluated *ABCG2* c.421C>A along with variations in other UA transporters (SLC17A3 and SLC2A9) in patients with Lewy Body Disorders (LBD). They did not find an association with *ABCG2* c.421C>A, but rather found that the "T" allele at the SLC17A3 SNP, rs1165205, was associated with higher CSF UA levels, and that subjects with LBD had lower UA levels in serum and CSF than controls.[139] However, UA baseline serum levels are age-dependent, and the population with LBD is generally older than study populations with TBI.

It is also plausible that the role of ABCG2 in regulation of amyloid-beta could explain some of our findings. A study by Jullienne and colleagues found amyloid-beta accumulation in their model for juvenile TBI.[185] The polymorphism *ABCG2* c.421C>A is thought to impact the regulation of amyloid-beta and may confer protection against Alzheimer's disease. Indeed, genetic risk factors for Alzheimer's disease (e.g. APOE $\epsilon 4$) are also risk factors for poor clinical outcomes from TBI, potentially related to similar amyloid-β plaque pathology.

3.4.4 Age and TBI Outcomes

Hukkelhoven and colleagues recently showed that advancing age independently predicts unfavorable outcome. They found an age threshold of 39 years old provided the best prediction for unfavorable outcomes and mortality.[7] Our findings provide supportive data that the recovery trajectory following TBI changes in the fourth decade of life. Future study will seek to explain the mitigation of genotype effect that happens in patients over the age of 34. As this explanation is speculative, it does provide insight into the potential changes in UA generation and metabolism with age. This might relate to changes in CNS plasticity and response to injury that occur at this age.

It is also possible that this is related to a change in microglia polarization (i.e. M1 v. M2 phenotype), though this area remains controversial.[186] The response to brain levels of UA may be age-related, wherein younger individuals might exhibit increased sensitivity to its role in the CNS antioxidant reserve. Finally, age-related differences in expression of compensatory mechanisms (i.e. other transport systems that compensate for ABCG2) might limit the benefit of having variant alleles. Full understanding this finding requires further mechanistic study into the biochemical disposition of patients suffering from TBI in the context of genotype at *ABCG2* c.421C>A.

3.4.5 Limitations

A limitation of our study is the single center study design. However, both groups were independently sampled at different time points, allowing for a classical experimental replication. Variation in fixed effects while showing the same association with similar magnitude across two heterogeneous cohorts is a strength of the findings, though it might challenge generalizability. Similarly, the interaction between genotype and age is interesting, but requires further study as it could result from population stratification. Finally, although replication of results in a separate cohort provides strong support of the finding, it is not possible to establish causation from a retrospective evaluation. Further investigation into the molecular mechanism associated with the genetic association might better explain the results of this preliminary study.

3.5 CONCLUSION

An association of *ABCG2* c.421C>A with outcomes following severe TBI was identified in a discovery and replication cohort. Mixed effects multiple ordinal regression with GOS scores as the dependent variable showed an age*genotype interaction, suggesting that having at least one ABCG2 variant allele was predictive of improved GOS score in subjects younger than 34, and of similar or lower GOS scores in subjects over 34 years old. ABCG2 function may impact brain recovery pathways after injury in an age-dependent manner.

4.0 IMPACT OF ABCG2 GENOTYPE ON CNS URIC ACID CONCENTRATIONS IN HUMANS POST TRAUMATIC BRAIN INJURY

4.1 INTRODUCTION

To explore the potential mechanism underpinning the findings in chapter 3, we sought to measure the disposition of the ABCG2 substrate, UA, in patients suffering from TBI. Increasingly, UA is understood to be a critical endogenous molecule. It has pathogenic qualities and is associated with metabolic disease, cardiovascular disease, and is the causative agent for gout.[187, 188] However, in aqueous environments it is a potent antioxidant and is critical for scavenging free peroxynitrite free radicals.[189]

4.1.1 Uric Acid: An Antioxidant with a Complex Past

As an endogenous product resulting from the breakdown of purines (**Figure 4.1**), Uric acid has roles as a pathogenic molecule that promotes cardiovascular disease through pro-oxidant effects and as a protective antioxidant in aqueous environments.[187] The major disease states with which UA is implicated are gout, urinary calculi and cardiovascular diseases.[187]

Most notable of diseases associated with UA is gout. Gout has been identified for millenia and was thought to be related to humoral imbalance.[190] Centuries ago, Garrod performed early lab testing for UA as a means of differentiating gout from rheumatoid arthritis.[191] Since that work, hyperuricemia has been confirmed as the precipitating factor of gout. This occurs as a result of UA rising above its level of solubility and the subsequent precipitation of monosodium urate in synovial fluid, prompting an inflammatory reaction that is alleviated by non-steroidal anti-inflammatory medications and colchicine.[192] Patients with cardiovascular disease tend to have higher levels of UA and risk for gout. Current understanding suggests that elevated UA may be a symptom of these illnesses, but not a causative agent.[188]

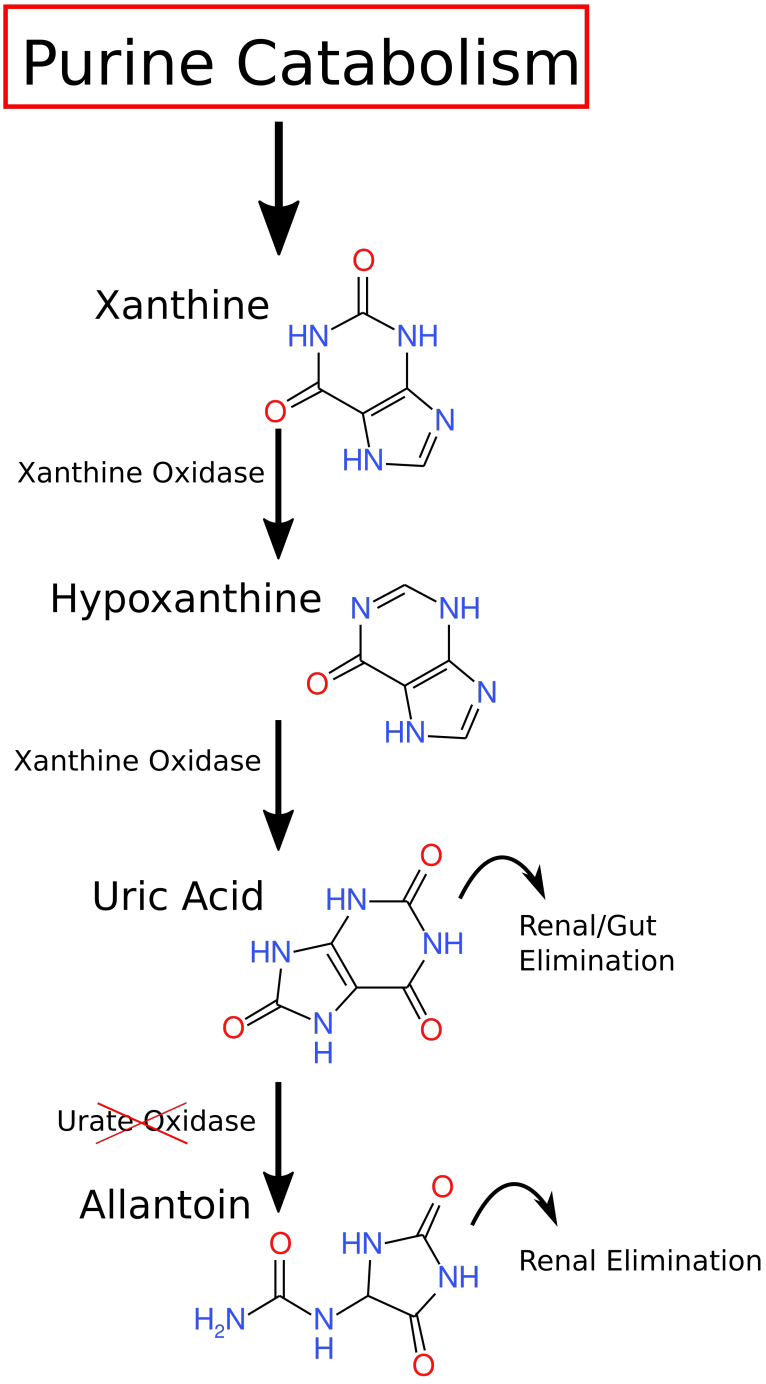

Figure 4.1: The purine metabolism pathway ends with uric acid in humans due to a mutation in urate oxidase. In most other mammals, allantoin is produced from uric acid by urate oxidase.

4.1.1.1 Evolutionary History of Uric Acid

Humans and other new-world hominids including chimpanzees, orangutans, and bonobos have much higher levels of serum UA than other mammals (and most other animals).[193, 194] This ties to a mutation in the urate oxidase gene that occurred during the Miocene era between 10 and 20 million years ago. Presence of non-functional urate oxidase is believed to be the precipitating factor for allowing higher serum levels of UA, which may have enabled larger, more metabolically active brains characteristic of these hominids.[194] It has also been hypothesized by Watanabe and colleagues that this could have conferred a survival advantage by allowing better salt and fluid retention, which may help also explain the perceived connection between UA and hypertension/cardiovascular disease.[195]

4.1.2 Potential of Uric Acid as a Mediator of Secondary Injury

Secondary injury is partly driven by increased reactive oxygen species (ROS) in the brain.[109] Uric acid (UA) is a component of the plasma and brain antioxidant reserve and is critical for peroxynitrite scavenging.[196, 189] The major renal and gut UA transporter, ABCG2, is expressed on the BBB and the blood-CSF barrier.[178, 177] It may be responsible for both the efflux of UA from brain to blood on the BBB and for the movement of UA from blood to cerebrospinal fluid blood-CSF barrier.[135] The common genetic variant in ABCG2, rs2231142 (c.421C>A), is associated with improved clinical outcomes following TBI, possibly due to increased brain concentrations of UA. ABCG2 c.421C>A is a missense mutation that causes decreased protein expression of ABCG2 and is a well-established risk factor for hyperuricemia, age of onset of Parkinson's Disease, and clinical outcomes following TBI.[119, 138, 163] A link between this variation and UA concentrations in the central nervous system (CNS) has not previ-

ously been established.

4.1.2.1 Uric Acid is a Free Radical Scavenger

Peroxynitrite free radicals and iron generated free radicals are generated in the brain following TBI.[197] Work by Keane and colleagues showed that UA's peroxynitrite scavenging may also support the integrity of the BBB, which may have relevance in TBI due to the common disruption of the BBB post severe TBI. Uric acid relies on the presence of ascorbic acid for the neutralization of peroxynitrite free radicals, and ascorbate is not able to scavenge peroxynitrite at all.[198] Interestingly, UA's antioxidant action is limited to aqueous environments, and it is not able to scavenge free radicals within lipid membranes.[198]

4.1.3 Objective and Hypothesis

Our objective was to measure UA levels in the CNS in the context of severe TBI and ABCG2 c.421C>A as a surrogate for transport capacity. We hypothesized that presence of the "CA" genotype at ABCG2 c.421C>A would be associated with decreased CSF UA post TBI, and that decreased CSF UA would be associated with improved clinical outcomes. Our expectation that UA would be decreased in the CSF is due to the opposing localization of ABCG2 on the BBB and blood-CSF barrier, wherein ABCG2 effluxes from blood to CSF on the blood-CSF barrier and from brain to blood on the BBB. To test this hypothesis, we needed to develop and validate a standardized method for determining the concentration of UA in clinical samples.

4.2 METHODS

4.2.1 Uric Acid Assay Development

4.2.1.1 Commercial Kit for Uric Acid Measurement

Measuring UA in biologic samples is not a novel undertaking. Uric acid has been measured in blood and urine in some sense for centuries.[191] We therefore approached the need to measure UA as pragmatically as possible, starting by evaluating the available commercial tests as they may apply to this study. Initially, we sought to us the widely available clinical tests which can be accessed through the hospital laboratory or through commercial kits, but this proved problematic. Nearly all clinical assays and commercial kits use enzymatic methods for determination of UA concentration. Most commonly, they rely on the oxidation of UA to allantoin by recombinant urate oxidase. In the presence of oxygen, this produces hydrogen peroxide, which converts a precursor molecule to a colored dye that can be measured colorimetrically.

The problems we encountered with assay methodologies that relied on oxidation of UA tied to the sample types we have. For the CSF samples, the expected concentration is very low (1-50μM), which is achievable with some kits, but we were skeptical of the reliability as there was no published literature where someone had used a kit to quantify CSF UA. Specifically, the Amplex® Red UA acid assay kit documentation suggests detection as low as 100nM of uric acid when used with the protocol for fluorometric measure.[199] While this lower limit is adequate for our CSF concentrations, we were unable to satisfactorily determine the expected performance when using CSF with this kit. In addition, many samples were tinged with blood, which can potentially interfere with the performance of the Amplex® Red reagent. The plasma samples

were also problematic for the commercial kits. Our bio-banked plasma samples were anticoagulated with EDTA, a calcium ion chelator. EDTA also functions as an antioxidant itself and can compete with the reagents for hydrogen peroxide. This could potentially bias the results, and would be difficult to correct for due to varying levels of EDTA in each sample (i.e. blood volumes vary in the same size tube). Due to these potential challenges, we sought to develop UA assays for EDTA plasma and CSF using high performance liquid chromatography tandem ultraviolet detection (HPLC-UV).

4.2.1.2 Characteristics of Uric Acid and Standard Preparation

Uric acid is a small molecule (168.112 g/mol) with very poor solubility (60 mg/L in water at 20°C). Its logP is -2.17, indicating that its solubility in non-polar solvents is even poorer than in water. This is illustrative of the pathologic causes of gout and urolithiasis - UA simply does not solvate well. This can be improved by the use of an alkaline solvent. Previously published options include an aqueous solution of lithium carbonate, potassium hydroxide, sodium hydroxide, and ammonium hydroxide.[200] Xinhua found that at a 1.7:1 molar ratio of ammonium hydroxide to UA provided sufficient alkalinity to solvate UA with no evidence for stability issues.[200] For plasma and CSF we need to be able to make standards ranging from 1 to 600μM, so a stock concentration of 600μM will be the minimum stock concentration to achieve. Given that at the 1.7:1 ratio, UA still takes a significant amount of time to go into solution - we opted to approximately double the ratio and make all UA stocks fresh with a molar ratio of 2mM ammonium hydroxide:0.6mM UA. There is, however; no evidence that this higher concentration of ammonium hydroxide causes instability of UA.

4.2.1.3 Selection of Internal Standard

Similar to work by Soto-Otero and colleagues, we used 7-(β-hydroxyethyl) theophylline (Etophylline), a theophylline derivative similar in structure to UA. This has been used as an internal standard for several purine derivatives in HPLC-UV assays, including caffeine.[201] It provides the needed molecular similarity, but with significantly different characteristics within a reverse-phase column that provides a much longer retention time than UA due to its increased lipophilicity. Specifically, UA is much more hydrophilic and will elute with the inorganic potassium phosphate buffer, whereas etophylline will elute with the acetonitrile. The structures of UA and etophylline are shown in **Figure 4.2**.

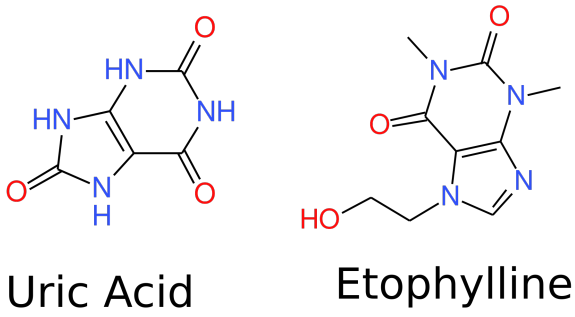

Figure 4.2: Uric acid and its assay internal standard, etophylline, are chemically similar and have been previously used together in HPLC-UV.[201]

4.2.1.4 Assay Reagents and Parameters

The CSF and plasma UA assays followed similar parameters as reported by Soto-Otero, with several modifications.[201] Both assays were run on a Waters 2695 separation module. UV detection was performed using a Waters 247 dual λ absorbance detector. The column used for both assays was a C_{18} 5μm 3.9mm X 150mm column (part num-

ber WAT046980, Waters Corporation, Milford, MA). Both assays used a 0.06M potassium phosphate buffer with 2% methanol (pH approximately 5.1) as the primary mobile phase with 100% HPLC grade acetonitrile with gradient. Stroke volume was 50μL for both assays. Also for both assays, absorbance was measured on two channels, 292nm for UA and 272nm for etophylline. For samples, both assays started with 60μL of plasma or CSF to be assayed (standards also 60μL), to which 240μL 100% acetonitrile was added to precipitate protein. Samples were vortexed for 30 seconds, then centrifuged at 10,000G for 10 minutes. Supernatents were transferred to glass tubes and dried under nitrogen at 60°C for approximately 15 mninutes. Samples were reconstituted with the primary mobile phase, covered with paraffin film, vortexed for 30 seconds, then transferred to 300μL glass HPLC sample tubes. The auto-sampler was maintained at 25°C for CSF and plasma samples.

CSF Assay

CSF standards were made in artificial CSF made with the reagents shown in **table 4.1** with a standard curve containing 1μM, 5μM, 10μM, 20μM, 50μM, and 100μM. Following preparation and loading into the auto-sampler, an injection volume of 100μL was made into the HPLC circuit. The column was maintained at 28°C at a rate of 0.75mL/min with a gradient flow that started with 100% Potassium Phosphate Buffer with addition of acetonitrile at a rate of 2% per minute for the first 2 minutes, then 3% per minute for an additional 7 minutes, and finally 2% per minute for the final minute leaving a makeup of 24% acetonitrile and 76% potassium phosphate buffer. This was followed by a return to 100% potassium phosphate buffer, which was run for an additional 5 minutes for washout and re-conditioning the column (15 minute runtime total). The standard curve was fit using the area ratio of UA to etophylline,

weighted using: $1/x^2$ in standard linear regression using R to minimize bias at the lower end of the curve.

Table 4.1: Artificial CSF Composition

Solute	Concentration (mM)
NaCl	119
KCl	2.5
NaH_2PO_4	1
$MgCl_2$	1.3
$CaCl_2$	2.5
Glucose	10

Plasma Assay

Plasma standards were made in phosphate buffered saline with a standard curve containing 60μM, 90μM, 120μM, 240μM, 480μM, and 600μM. Following preparation and loading into the auto-sampler, an injection volume of 50μL was made into the HPLC circuit. The column was maintained at 35°C at a rate of 1mL/min with a gradient flow that started with 100% Potassium Phosphate Buffer with addition of acetonitrile at a rate of 2% per minute for the first 2 minutes, then 3% per minute for an additional 5 minutes, followed by a return to 100% potassium phosphate buffer, which was run for an additional 3 minutes for washout and re-conditioning the column (10 minute runtime total). The standard curve was fit using the area ratio of UA to etophylline linear regression with no weighting applied using R.

4.2.2 Uric Acid Stability

4.2.2.1 Rationale

The samples used within this study have been stored consistently at -80°C and -20°C since collection. While UA is thought to be stable in clinical serum samples for at least

a year, we were worried about the integrity of the UA in the samples since several had been stored for over ten years. There is also little or no data about the long-term stability of UA in EDTA plasma or CSF. We therefore carried out two stability studies in CSF and in EDTA plasma. This was done in the respective biological matrices, spiked with UA stock solution to produce a high and a low concentrations.

4.2.2.2 Sample Preparation

Briefly, 4mL of pooled EDTA plasma and 4mL of pooled CSF was collected in separate 10mL centrifuge tubes. Pooled samples were vortexed for approximately 10 seconds, then split (2mL each) into separate centrifuge tubes. A 1mM UA stock solution was prepared in 2mM ammonium hydroxide. This was diluted to 150μM for the CSF. Finally, 400μL of the 150μM stock solution was added to the high CSF sample, and 400μL of the 1mM stock solution was added to the high plasma sample. For the low CSF and plasma samples, 150μL of 2mM ammonium hydroxide were added. Spiked stocks were aliquoted into three replicates for both levels for each planned assessment. We did not assess bench-top stability as that is not an expected storage condition of these samples. The same stock solution was assayed after being created for a time 0 concentration for use as a comparator.

4.2.2.3 Free-Thaw Stability

Freeze thaw stability was assessed based on the recommendations in FDA Bioanalytical Method Validation Industry Guidance.[202] Three replicates of the high and low concentration in plasma and CSF were stored at -20°C and thawed at room temperature on the bench-top for 1-2 hours, then allowed to re-freeze for at least 24 hours. This was repeated three times in a two week period, then re-assayed.

4.2.2.4 Storage Stability

Storage stability was assessed after continuous freezing at -20°C for 14 days and 2 months. Replicates at each level were pre-labeled for their destined measurement time point. The maximum time point of two months was chosen due to practical concerns with timely completion of this data, but additional aliquots are frozen and available for later testing.

4.2.3 Subjects and Samples

4.2.3.1 Population

Subjects (N=41) for this study were selected from the population described in chapter three (severe, non-penetrating TBI). The goal of this study was to identify the potential mechanistic links of the genotype association with outcome, thus we sought to enrich the population. While this is a non-random design, it increases statistical power (theoretically) by increasing the comparator sample size, thus decreasing the standard deviation of the measure in the comparator group. Subjects were selected from the first (discovery cohort), which did not have any individuals homozygous for the "A" allele at *ABCG2* c.421C>A so we focused on getting two nearly equal groups with 1) near complete or complete outcomes data and 2) equal numbers of "CC" genotypes and "CA" genotypes. This artificially inflates the minor allele frequency from an expected 0.12 to 0.25. As with the previous study, Glasgow Outcome Scale (GOS) scores were collected at 3, 6, 12, and 24 months post injury and dichotomized to poor outcome (1-3) or good outcome (4-5).

4.2.3.2 Plasma and CSF Samples

The first goal of this study was to assess the impact of genotype at *ABCG2* c.421C>A

on UA in the CNS. Thus, subjects were only included if they had available bio-banked CSF collected from bagged drainage from extra-ventricular drains for part of their hospitalization. Though not a requirement, we also collected bio-banked plasma from patients for use as a contrast and a means to calculate surrogate transport capacity. For plasma and CSF, variable numbers of samples were available per patient. We measured each sample for CSF and the first available plasma sample using the validated HPLC-UV assay. Plasma samples that had concentrations below the level of quantification on the plasma assay were re-run on the CSF assay.

Due to the presence of hemorrhage in many patients suffering from severe TBI, blood contamination is common within CSF. This raises the possibility of artificially high levels of CSF UA due to the much higher concentration of UA in the blood. To correct for this, we used a visual scale ranging from 0 to 2, with 0 = no blood (colorless), 1 = blood tinge (slightly red or pink), and 2 = frank blood (very red). This was evaluated with concentration using linear regression to determine if it was appropriate to apply a correction to the measured concentrations. If blood contamination shows significant association with UA concentrations, a correction factor will be applied to the raw concentration values by subtracting the coefficient multiplied by the assigned value from the visual blood scale.

4.2.4 Statistical Methods and Data Analysis

All analyses were completed using R v3.4.3 (R Development Core Team, Vienna, Austria).

4.2.4.1 Assay Validation

Assay validation was performed under the guidance of the FDA Bioanalytical Method

Validation Industry Guidance.[202] Plasma and CSF assays were separately validated, with no more than one validation run happening in a 24 hour period. Each validation run consisted of a standard curve run in duplicate. For inter-day validations (2), low, mid, and high quality controls were run with six replicates. For the intra-day validation (1), quality controls were ran with twelve replicates. Validation was determined based on QC passes within the FDA recommended guidelines for inter- and intra-day variations (20% for low, 15% for medium and high). Lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were determined from the lowest and highest points on the standard curve, respectively. These were performed in duplicate on each validation day for a total of six replicates measured throughout the entire validation process. We did not assess for matrix effects, recovery efficiency, or process efficiency, though it is acknowledged that these could have been carried out.

4.2.4.2 Direct Stability Studies

Per the FDA guidance, samples were run in triplicate for each measurement group. Nominal concentrations for each level were taken from the day zero assay, which immediately followed preparation of the high and low standards. These were compared with measurements at the specified time points. Acceptance of the results and assumption of adequate stability was determined by the presence of a bias of at most 15%.

4.2.4.3 Long Term Stability (Indirect)

To bridge the findings in the relatively short term stability study, we used the existing samples available for the study to indirectly measure the stability trend. This was carried out by fitting a regression equation to all plasma and CSF data points (separately)

versus time. While individual differences exist between subjects, the slope should not deviate substantially from zero. This provide an indirect measurement of extremely long term (5+ years) of storage and freeze/thaws.

4.2.4.4 Descriptive Data

Population demographics and outcomes are compared with T test for normally distributed data and chi-square for categorical data. Data are presented as median +/- interquartile range or as frequencies.

4.2.4.5 CSF Uric Acid Concentration and Genotype

CSF comparisons were based on the maximum measured UA concentration in a subject's set of samples. This was done to minimize bias induced by some subjects having extended time periods with samples vs. some with one or very few samples. Maximum CSF UA levels were compared across genotype at ABCG2 c.421C>A and evaluated with multiple linear regression controlling for age and sex.

4.2.4.6 CSF Uric Acid Concentrations and Outcomes

Max CSF UA was tested as a predictor of TBI clinical outcomes with mixed effects logistic regression controlled for age, GCS, and time post TBI allowing for a random intercept on subject. Due to the potential correlation between genotype and UA concentrations, genotype was not included in this model. The outcome variable was dichotomized to enhance model simplicity and ease of interpretation. This was accomplished by coding GOS 1-3 as an unfavorable outcome, and GOS 4-5 as a favorable outcome.

4.2.4.7 Surrogate Transport Capacity

Due to the expression pattern on the blood-CSF barrier versus the BBB, CSF UA was assumed to be an inverse surrogate for brain UA. We calculated a rough estimate of transport capacity using the plasma:CSF UA ratio, where higher numbers indicate decreased transport of UA. A single plasma concentration measurement from patients was used to calculate this measure. This measure was compared across genotype controlled for age and sex with linear regression. It was also compared with outcomes using mixed effect linear regression with the same covariates and random effects as the CSF UA concentrations compared with outcomes.

4.3 RESULTS

4.3.1 Uric Acid Assay Validation

Both assays were validated and run on the same column several months apart. For the CSF assay validation, there was one QC excluded in the 90μM inter-day runs, one exclusion in the 45μM inter-day runs, and one exclusion in the 45μM intra-day run. For the plasma assay validation, there was a single exclusion in the 75μM inter-day. All other parameters passed within the FDA recommended criteria (**Table 4.2**). Illustrative chromatograms are shown for the CSF (**Figure 4.3**) and the plasma (**Figure 4.4**).

Table 4.2: Assay Validation Results

(a) CSF

Concentration	2μM	45μM	90μM
	Inter-Day		
Mean	1.97	44.9	90.83
SD	0.14	2.18	2.75
CV	7.11%	4.86%	3.03%
Bias	-1.50%	-0.22%	0.92%
	Intra-Day		
Mean	1.86	44.24	89.64
SD	0.09	2.42	1.26
CV	4.84%	5.47%	1.41%
Bias	-7.00%	-1.69%	-0.40%

(b) Plasma

Concentration	75μM	300μM	585μM
	Inter-Day		
Mean	73.92	297.56	578.78
SD	1.83	5.03	13.29
CV	2.47%	1.69%	2.30%
Bias	-1.44%	-0.81%	-1.06%
	Intra-Day		
Mean	73.55	299.17	572.42
SD	2.12	5.30	12.47
CV	2.89%	1.77%	2.18%
Bias	-1.94%	-0.28%	-2.15%

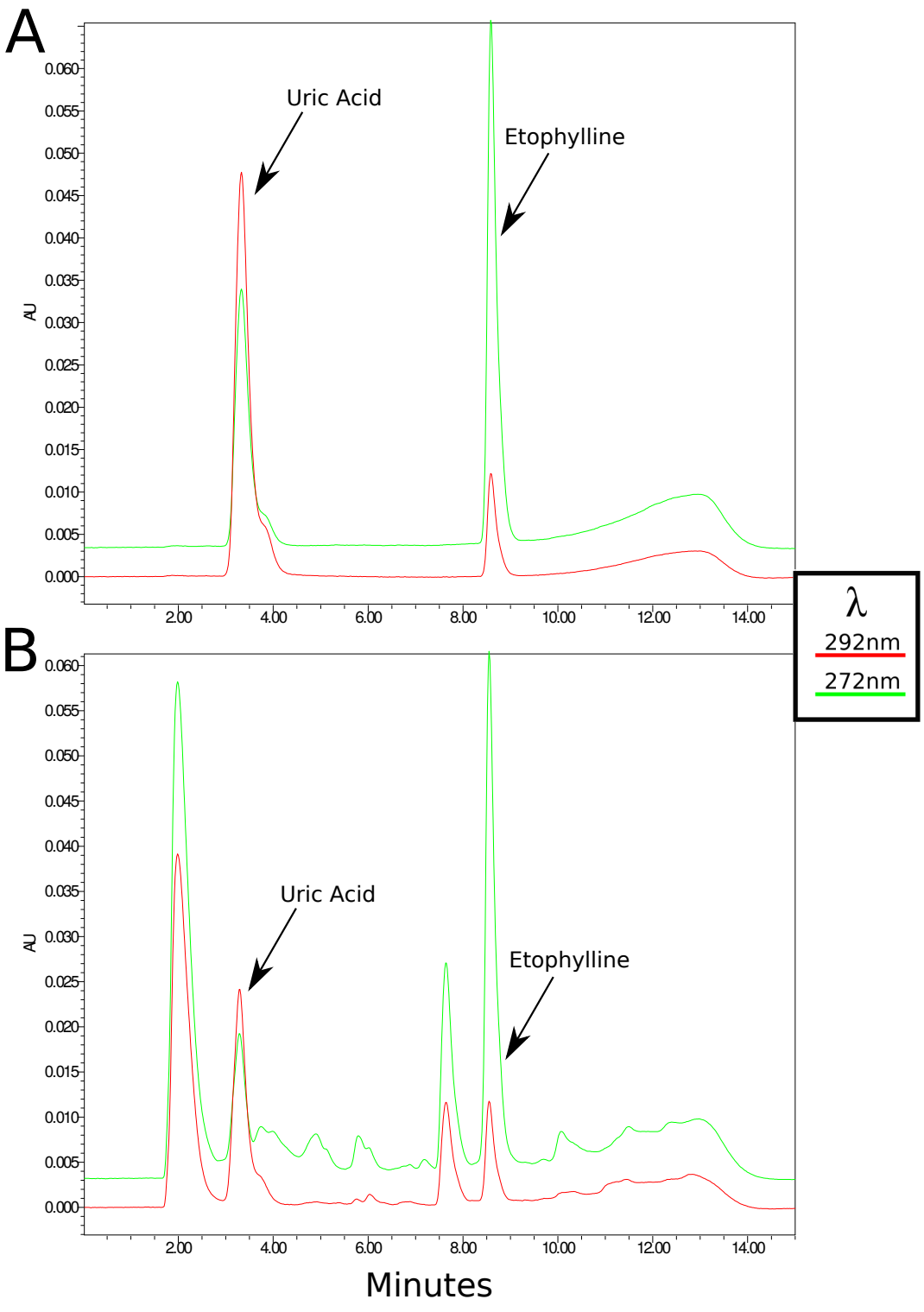

Figure 4.3: Panel A shows a representative chromatogram for a CSF 50μM standard. Panel B shows a representative unknown CSF sample.

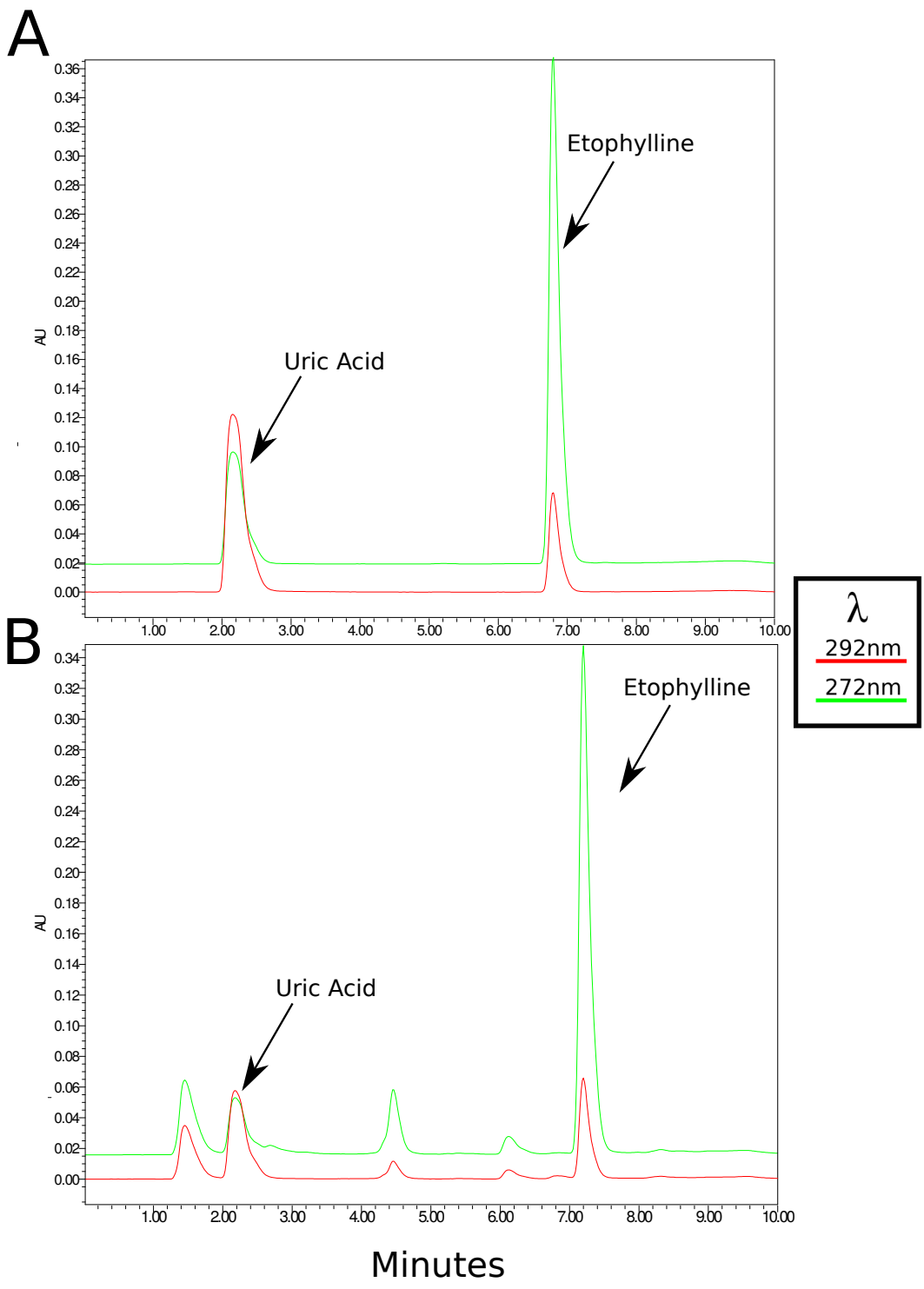

Figure 4.4: Panel A shows a representative chromatogram for a plasma 240μM standard. Panel B shows a representative unknown plasma sample.

Assessing the standard curves from the validation runs showed linearity as low as 1μM for the CSF assay, and 60μM in the plasma assay, up to the maximum concentration on each assay. All standard curve values performed within 15% of the expected concentration. This suggests a LLOQ of 1μM in CSF and 60μM in plasma, and an ULOQ of 60μM in the CSF and 600μM in the plasma (**Table 4.3**).

Table 4.3: Assay Standard Curve Performance

(a) CSF

Concentration	1μM	5μM	10μM	20μM	50μM	100μM
Mean	0.99	5.1	10.07	20	50.71	96.5
SD	0.07	0.21	0.41	0.66	1.72	1.63
CV	7.41%	4.14%	4.04%	3.28%	3.39%	1.69%
Bias	-0.57%	2.05%	0.75%	0.00%	1.41%	-3.50%

(b) Plasma

Concentration	60μM	90μM	120μM	240μM	480μM	600μM
Mean	61.02	91.01	118.97	238.6	479.41	600.98
SD	1.5	1.21	1.89	4.14	5.65	5.43
CV	2.46%	1.32%	1.59%	1.73%	1.18%	0.90%
Bias	1.70%	1.12%	-0.86%	-0.58%	-0.12%	0.16%

4.3.2 Uric Acid Stability

The baseline measurement in the CSF standard were 15.94±0.14μM for the low, and 40.11±0.88μM for the high. The baseline measurement in the plasma standard were 148.35±4.42μM for the low, and 237.42±19.06μM for the high. This shows that the unadulterated initial pooled CSF concentration was 19.13μM based on [$(15.94 * 2400)/2000 = 19.13$] and the unadulterated initial pooled plasma concentration was 178.02μM based on [$(148.35 * 2400)/2000 = 178.02$]. These initial concentrations

also support the spiked concentration, showing an estimated concentration of 40.94μM in the high CSF (2.1% higher) and 315.01μM in the Plasma (32.7% higher). The measured versus the expected concentration in the high plasma concentration are higher than ideal, but not unexpected based on the challenges in creating a 1mM stock UA solution. The transfer of fluid into the 2mL aliquot may also have been inaccurate. Regardless, adherence to expected concentrations is not critical for this step - but rather the consistency in measurements for downstream tests.

The freeze thaw study proceeded without issue. The maximum deviation from the time0 concentration was a 5% lower reading in the plasma low concentration. A summary of the findings from the freeze thaw study are shown in **table 4.4**.

Table 4.4: Freeze thaw stability comparisons between time point 0 and the two week time point following three freeze thaws.

Level	Plasma			CSF		
	Mean (μM)	SD	Bias	Mean (μM)	SD	Bias
Low0	148.35	4.42	0.00%	15.94	0.14	0.00%
Low	140.24	2.58	-5.47%	15.45	0.17	-3.12%
High0	237.42	19.06	0.00%	40.11	0.88	0.00%
High	233.89	3.77	-1.49%	38.46	1.41	-4.10%

low0 and high0 refer to the initial concentrations assayed on day 0

The -20°C storage stability was completed up to 60 days following the initial freeze. No measurements deviated beyond the acceptable limit of 15%. The findings are summarized in **table 4.5**.

Table 4.5: Uric Acid Stability Stored at -20°C for 14 and 60 days.

Level	Plasma			CSF		
	Mean (μM)	SD	Bias	Mean (μM)	SD	Bias
Low0	148.35	4.42	0.00%	15.94	0.14	0.00%
Low 14d	144.78	7.79	-2.41%	15.22	0.15	-4.53%
Low 60d	134.83	6.59	-9.11%	15.25	0.45	-4.33%
High0	237.42	19.06	0.00%	40.11	0.88	0.00%
High 14d	235.61	7.02	-0.76%	39.16	0.78	-2.36%
High 60d	233.88	3.74	-1.49%	40.74	0.79	1.56%

low0 and high0 refer to the initial concentrations assayed on day 0

Findings from the long term stability study are shown in **Figure 4.5**. Each year was associated with a non-significant 0.97μM decrease in UA in the CSF (p=0.063), and a 0.96μM decrease in the plasma (p=0.211). These suggest that there was not a problem with UA stability in long-term storage.

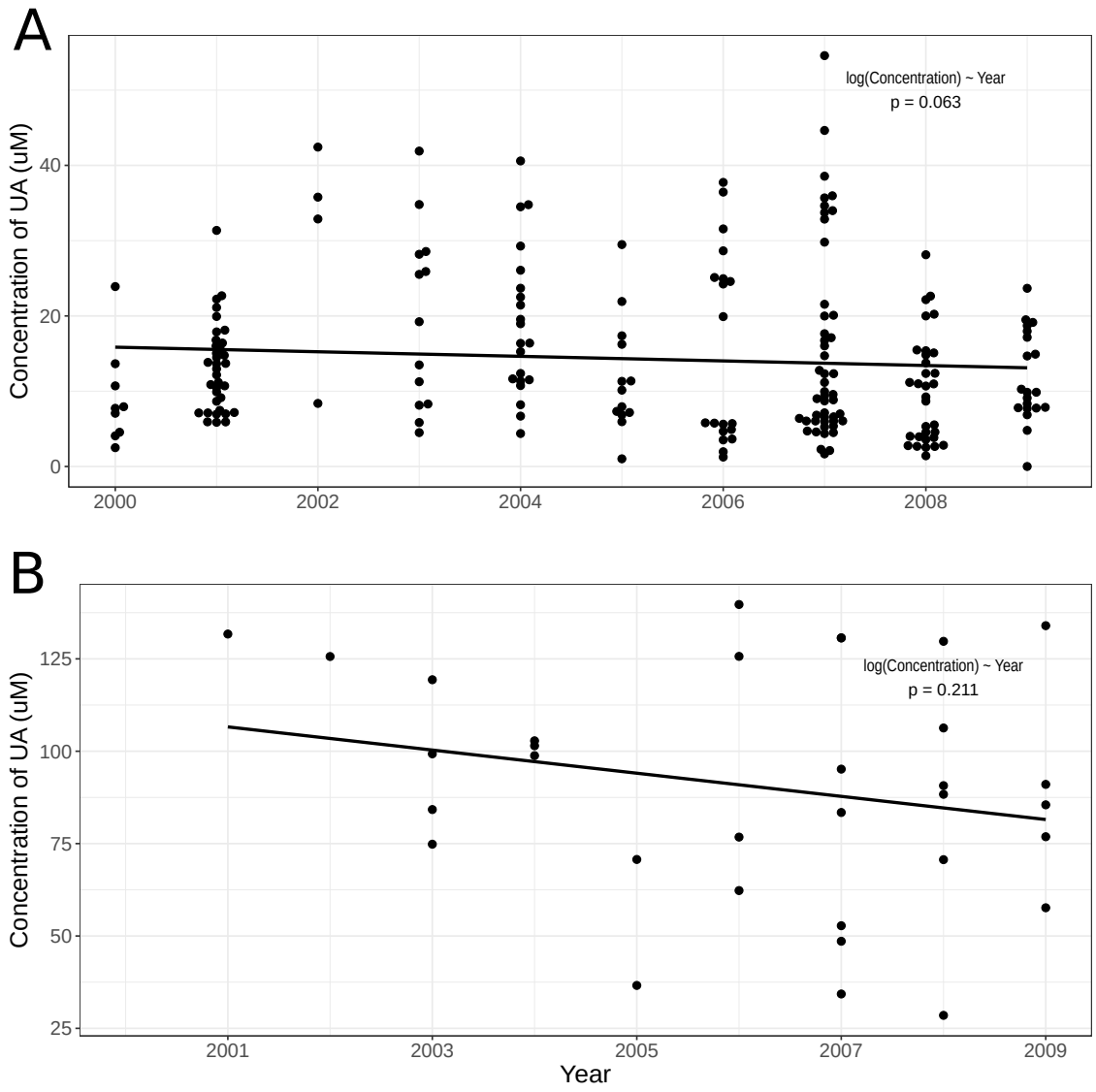

Figure 4.5: Panel A and B show all CSF and plasma levels assayed plotted against the year they were collected, respectively. P values reflect the association between year and concentration, suggesting that time is not associated with a significant change in the average concentrations obtained.

4.3.3 Population Demographics

Of the 41 subjects, all had at least one CSF sample available and 33 had a plasma sample available. 68.3% of the subjects were male. The median ± IQR age in years of the cohort was 25 +/- 19. Split by genotype, the cohort did not significantly differ in age, sex, or GCS score. Demographics by genotype are summarized in **Table 4.6**.

Table 4.6: Cohort Demographics

Factor	c.421C>A CC	c.421C>A CA	p value
Age Median (IQR*)	25 (20.75-41.5)	25 (21-32)	0.88
Female (%)	35.00%	28.60%	0.92
GCS** Median (IQR*)	6 (6-7)	7 (6-7)	0.89
Hemorrhage Type			
None	1 (5%)	0 (0%)	0.98
Epidural Hematoma	1 (5%)	5 (24%)	0.16
Subdural Hematoma	10 (50%)	8 (38%)	0.65
Subarachnoid Hemorrhage	9 (45%)	7 (33%)	0.66
Intraparenchymal Hemorrhage	4 (20%)	6 (29%)	0.78
Intraventricular Hemorrhage	2 (10%)	6 (29%)	0.27
Diffuse Axonal Injury	2 (10%)	2 (10%)	1.00
No Imaging Data	4 (20%)	6 (29%)	0.78

4.3.4 CSF Uric Acid

Among the 41 subjects, 213 CSF samples were available. Raw data are plotted by individual and by genotype in **Figure 4.6**

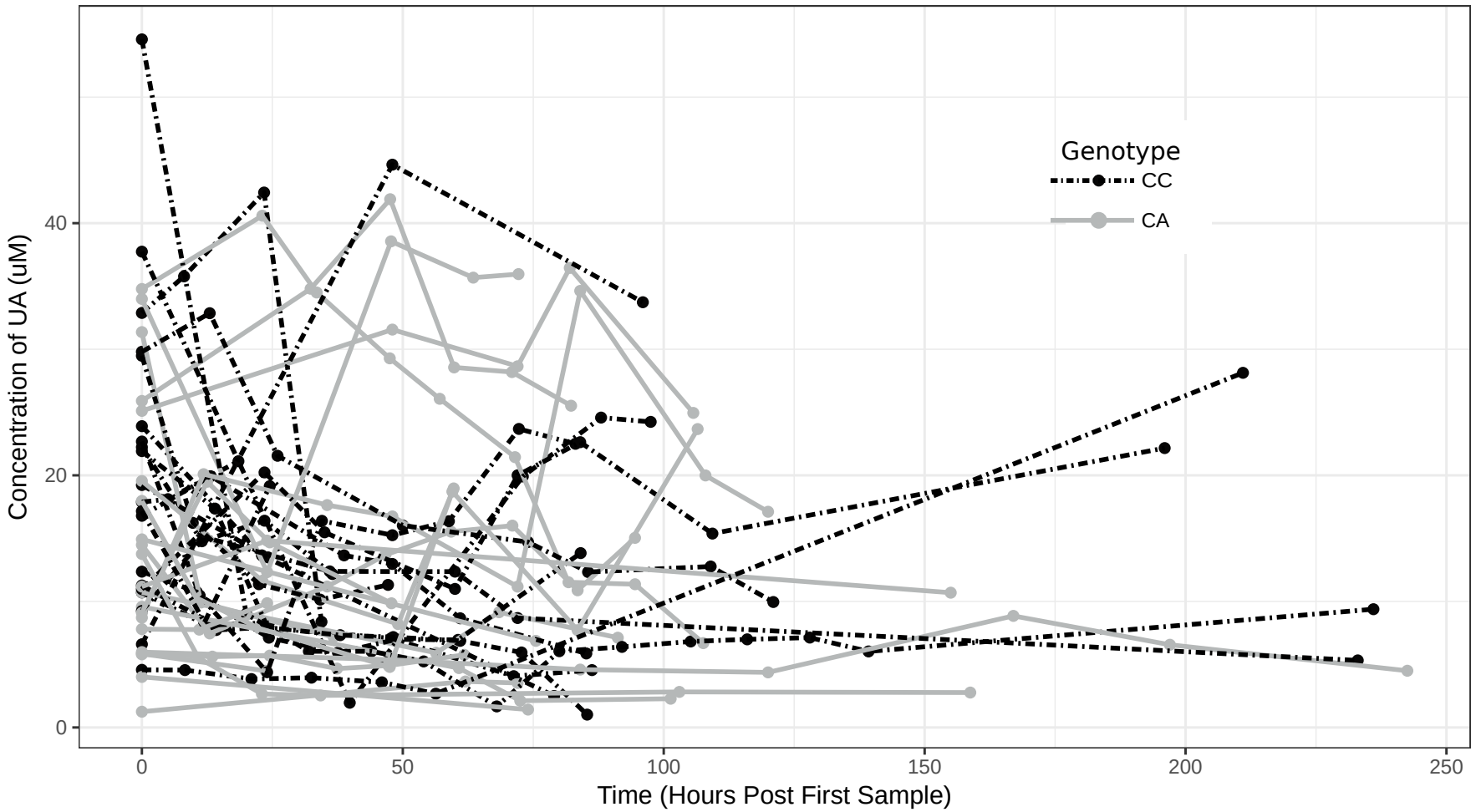

Figure 4.6: Uric Acid Concentrations in the CSF concentrations split by genotype at *ABCG2* c.421C>A.

Utilizing all CSF UA concentrations, we found that each level increase in the blood scale was associated with a 4.53μM increased concentration (p=0.0003). Therefore, a statistical correction was applied to the CSF concentrations to account for this. All subsequent measurements using the CSF UA reflect the corrected values. The median maximum UA ± IQR UA in the CSF was 19.37uM ± 14.93 in the CSF. Presence of the "CA" genotype at ABCG2 c.421C>A was associated with significantly lower CSF UA (p=0.03, **Figure 4.7**).

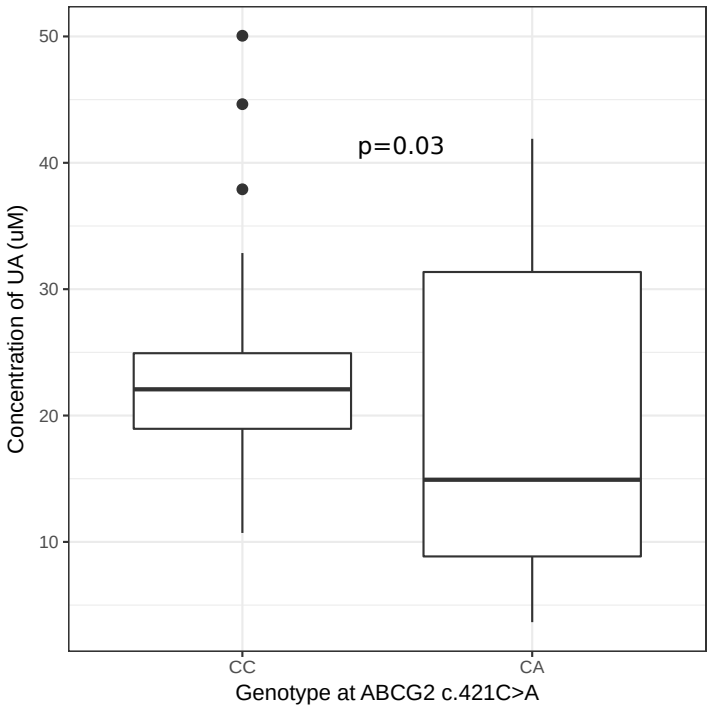

Figure 4.7: Uric Acid Concentrations in the CSF split by genotype at *ABCG2* c.421C>A. The median ± IQR in the CC group was 22.07 ± 5.98 veresus 14.92 ± 22.51 in the CA group.

When controlling for age and sex, the association of CSF UA and genotype was

maintained (p=0.022), suggesting that patients with the "CA" allele had 4.50μM lower concentration in comparison to those the the "CC" genotype. Considering the age * genotype interaction found in chapter 3, we evaluated this model with a genotype * age interaction. This showed a significant genotype (p=0.001), and genotype*age interaction (p=0.01) effects. The predicted CSF UA concentration with age is shown in **Figure 4.8**.

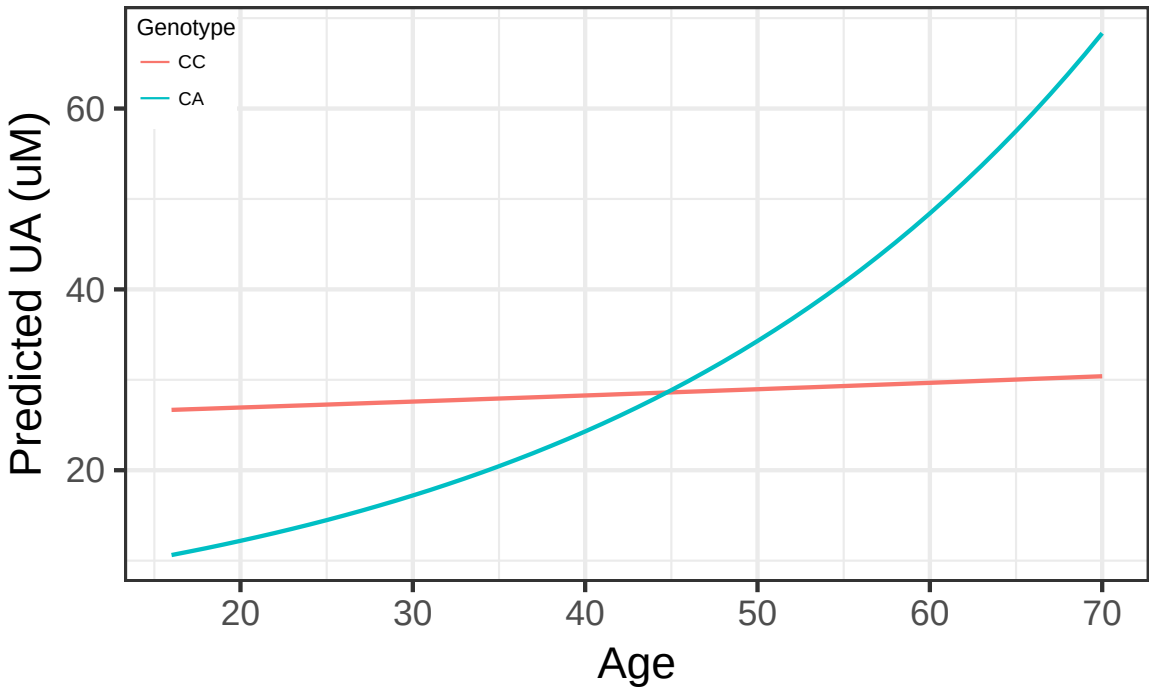

Figure 4.8: Uric Acid concentrations plotted with increasing age to illustrate the impact of the interaction between genotype an age. This is a log-linear approximation and may not necessarily reflect predictions at extremes.

Due to the relatively small sample size and the risk of over-parameterizing the model, we did not correct for bleed patterns in the outcomes models. This decision was made due to repeated failure of model convergence, despite numerous attempts to rescale variables. The final outcomes model was carried out using only scaled age,

maximum CSF UA, time post injury, and a random effect based on subject ID allowing for random intercepts. We found that concentration of UA in the CSF was associated with decreased odds of having a favorable outcome (OR : 0.661, p = 0.021). This suggests that for every 1μM increase in CSF UA is associated with 33.9% lower odds of having a favorable outcome at any point following TBI. The results from this model are shown in **Table 4.7**

Table 4.7: Mixed Effect Logistic Regression for CSF Uric Acid and Outcomes

Factor	Odds Ratio	p value
Max UA Concentration	0.661	0.021
Age	1.00	0.970
GCS	0.767	0.761
Time Post Injury	1.35	0.009
Subject (Random)	NA	1

4.3.5 Plasma Uric Acid

Among the 41 subjects, 33 had at least one plasma sample available. The median ± IQR UA concentration was 90.69uM ± 48.64 in the entire population. Higher UA levels in the plasma were associated with higher max CSF UA concentrations (β=0.17, p=0.017), suggesting that an increase in 1μM UA in the plasma is associated with a 0.17μM increase in the CSF. Plasma concentrations are plotted by genotype in **Figure 4.9**.

Table 4.8: Mixed Effect Logistic Regression for Plasma Uric Acid and Outcomes

Factor	Odds Ratio	p value
Plasma UA Concentration	0.97	0.485
Age	0.89	0.361
GCS	0.77	0.814
Time Post Injury	1.32	0.062
Subject (Random)	NA	1

There was no significant difference in plasma concentration between genotypes (p=0.7004), age (0.256), or sex (p=0.426). Plasma concentrations were also not associated with outcomes when switched with max CSF UA in the previous model. Results from this study are summarized in **Table 4.8**. Note that the age variable was not scaled in this model to allow for convergence.

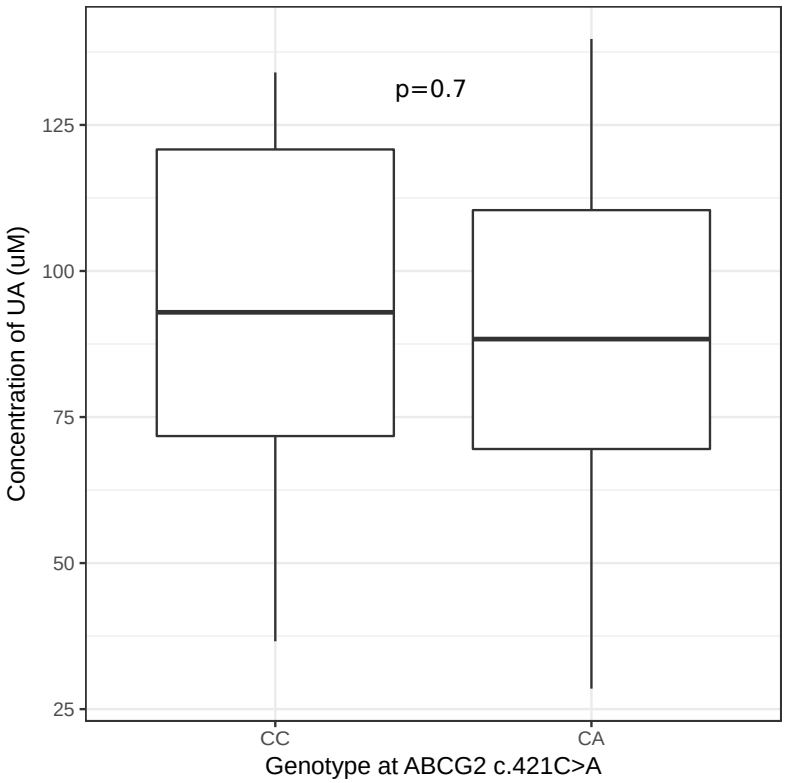

Figure 4.9: Uric Acid Concentrations in the Plasma split by genotype at *ABCG2* c.421C>A. The median ± IQR in the CC group was 92.93 ± 49.06 veresus 88.34 ± 40.88 in the CA group.

4.3.6 Surrogate Transport Capacity

The median concentration ratio ± IQR was 0.23 ± 0.19. Right skew was corrected with log transformation. When controlling for age and sex, the "CA" genotype at *ABCG2* c.421C>A was associated with a decrease in concentration ratio of 0.071 (p=0.045). This corresponds to a 35.6% decrease from the "CC" genotype. Beginning with a similar model as used for CSF uric acid with outcomes, we found that the scaled log of the concentration ratio was associated with higher odds of having a favorable outcome

(p=0.034). Results from the complete model are shown in **Table 4.9**.

Table 4.9: Mixed Effect Logistic Regression for CSF:Plasma Uric Acid and Outcomes

Factor	Odds Ratio	p value
log(Concentration Ratio)	0.007	0.034
Age	0.98	0.789
GCS	1.05	0.962
Sex	0.76	0.909
Time Post Injury	1.33	0.013
Subject (Random)	NA	1

4.4 DISCUSSION

In this study, we sought to evaluate the association between UA disposition in the CSF and plasma with genotype at *ABCG2* c.421C>A in patients suffering from severe TBI. Within that study, we developed an HPLC-UV assay for UA, which was validated in CSF and EDTA plasma. We also assessed the short term, freeze-thaw, and long term stability of UA in plasma and CSF, which showed that UA is likely stable enough for research purposes for at least five years. In our study of UA in the CSF, we found that lower CSF UA concentrations were associated with presence of an "A" allele at *ABCG2* c.421C>A. We also found that decreased concentrations were associated with outcomes. Finally, we found that decreased ratio of UA in the CSF to plasma was associated with presence of an "A" allele at *ABCG2* c.421C>A.

4.4.1 Uric Acid Assay and Stability

While successful methodology for the measure of UA has been available for over a century, the commercialized methods rely on relatively small concentrationrange of

samples.[203] Our development of an assay specific for EDTA plasma mitigates the issues associated with assays that rely on oxidation of UA for measurement. In addition, the use of UV detection with HPLC makes this assay able to measure lower levels and has much less cost than using mass spectrometry.

Numerous studies have been conducted to evaluate the short term stability of UA in clinical samples.[204, 205] These have generally found evidence that in most samples, UA demonstrates stability as far as 30 days post collection. Gislefoss and colleagues measured the stability of serum UA as far as 25 years after freezing and noted that the measured levels may be nearly 8% lower than at baseline. They suggest that much of the stability issue with UA relates to sample handling.[206] Our findings suggest that our storage conditions are adequate for stability, but it is important to acknowledge the benefit of using fresh tissue samples when possible. Our samples are also potentially protected by the presence of EDTA, a preservative that might improve the stability of UA.

4.4.2 Uric Acid Association with Genotype

4.4.2.1 Uric Acid and Serum/Gout

Uric acid has been studied extensively within the context of genotype at *ABCG2* c.421C>A. In the PAGE study, Zhang and colleagues found that presence of the "A" allele at *ABCG2* c.421C>A was associated with an increase of 0.24mg/dL of serum UA.[207] Cheng, et al found that the "CA" genotype and "AA" genotypes were associated with a 0.35 and 0.56mg/dL increase in serum UA, respectively.[208] These couple with the well studied and replicated association of *ABCG2* c.421C>A with hyperuricemia and gout following injury.

We did not replicate the association of UA in the plasma with *ABCG2* c.421C>A.

Previous investigations have shown that the "A" allele at *ABCG2* c.421C>A is associated with an increase of 18.44μM UA in plasma (European-American population).[207] Our population had a mean plasma UA of 90.45μM and standard deviation of 31.26μM. A rough power calculation suggests an effect size of 0.59 (18.44/31.26) would require at least 46 subjects to achieve 80% power. This suggests that our study was underpowered to show the association of genotype with plasma UA concentration.

4.4.2.2 Uric Acid in the CSF

Notably, the association of *ABCG2* c.421C>A in serum UA has not been shown previously in the CSF. Maetzler and colleagues investigated the levels of UA in the serum and CSF of patients with Lewy Body Disorders and did not find an association with genotype and CSF UA levels.[139] Notably, they also did not achieve adequate power to show a significant association with *ABCG2* c.421C>A an serum UA, either.[139] In our study, we found a significant association with the "CA" genotype at *ABCG2* c.421C>A and CSF UA, but not plasma UA. Notably different in our population is the age of the subjects - which is around 10 years less on average than many of the gout populations. Uric acid levels change significantly with age, and the other associations with *ABCG2* c.421C>A and UA are exclusively in older populations. This is further supported by the notion of age in our studies of *ABCG2* c.421C>A, wherein we have found that the associations with outcomes are limited to younger subjects.

4.4.3 ABCG2 Localization and Uric Acid Concentrations

4.4.3.1 ABCG2 on the BBB versus the blood-CSF barrier

These findings raise an important question, as we have consistently argued that the "A" allele at *ABCG2* c.421C>A is associated with better TBI outcomes due to *higher*

levels of UA in the brain, thus better antioxidant reserve. Our finding that 1) the "A" allele predicts lower concentrations in the CSF and that 2) lower concentrations in the CSF are associated with better TBI outcomes seem to contradict this over-arching hypothesis. However, this is most likely explained through the differences in localization of the ABCG2 transporter on the BBB and the blood-CSF barrier. On the BBB, ABCG2 is well known to face the blood, thus effluxing substrates from the brain to the blood. On the blood-CSF barrier, ABCG2 is likely CSF facing. This has been found in rodent studies of transporter localization carried out by Tomioka and colleagues.[135] Opposing directionality suggests that ABCG2 dysfunction could predict a decrease in the concentration in the CSF relative to plasma, and an increase in the brain relative to plasma (i.e. inverse relationship between CSF and brain). Of note, this does not mean that a drop in CSF UA corresponds to an increase in brain UA, but that the relationship of UA concentrations in the brain and CSF are inverted with respect to the contribution of ABCG2. Regardless of ABCG2, higher levels in the plasma ought to correlate to higher levels in both the brain and in the CSF.

4.4.3.2 Applying Decreased Transport Capacity with Localization

We found that decreased ABCG2 function, indirectly measured through the ratio of CSF to Plasma UA levels, was associated with the "CA" genotype at *ABCG2* c.421C>A. This supports the change in directionality, as one would expect that decreased function of ABCG2 would lead to less transport of UA from the blood to the CSF. This suggests, but does not directly measure or prove, that there is decreased removal of UA from the brain to blood in those with the "CA" genotype.

4.4.4 Limitations

This study is intended to build a mechanistic argument for the genetic association between *ABCG2* c.421C>A and outcomes following TBI. It is based on bio-banked samples collected incidentally, without purpose for this study. It is possible that the findings are based on chance variations in UA concentrations that are driven by sample age and/or storage conditions. Our selection process was focused on identifying patients who had complete data, CSF available, and at least one variant allele at *ABCG2* c.421C>A. This suggests that our findings could be influenced by selection bias, which could be improved by using a larger cohort of randomly selected subjects. We also note that our decision to limit analysis to the maximum CSF UA and a single plasma concentration time point was due to practicality and the expectation that 1) plasma levels would not vary substantially, and 2) incorporation of later CSF samples would bias the analysis to the lower baseline UA versus the relatively high levels found initially after injury. A more robust approach would be to collect CSF and plasma at the same time for multiple time points, which was not possible in this case. Our subject sampling strategy was done to intentionally enrich the frequency of variant alleles and to capture subjects with relatively complete data (e.g. outcomes collected at each time point). This strategy theoretically narrows the variations and increases power, but is non-random and inherently biased. While we do not expect that this drives the findings, it does suggest that following up with a larger population with newer samples would be prudent. Finally, because all data is retrospective and observational, we cannot say with certainty that UA is the mechanism by which ABCG2 influences outcomes. In fact, each factor identified could be incidental or a product of confounding. This is an inherent limitation of this study strategy, and countering these issues would

be impractical in a prospective randomized environment. Finally, we are limited to a clinical sample due to the differences in UA disposition in animals commonly used for TBI models. Therefore, we will hold to the argument that this methodology is the only practical way to carry out this study.

4.5 CONCLUSION

We evaluated the disposition of UA in the CSF and plasma in patients suffering from TBI. Subjects with the variant "A" allele at *ABCG2* c.421C>A, which confers decreased protein expression of ABCG2, were found to have lower concentrations of UA in the CSF and a decreased ratio of CSF:plasma UA. Finally, these changes in UA disposition in the CSF were associated with outcomes following injury. Overall, this study provides evidence for the use of UA and/or ABCG2 as a treatment target following TBI.

5.0 CONCLUSION

5.1 KEY RESEARCH FINDINGS

5.1.1 Transporter Expression Decreases Acutely Following Experimental TBI

The first study described in this dissertation was a study of transporter mRNA expression following experimental TBI with the controlled cortical impact model for TBI. Experimental TBI was carried out in 17 day-old rats compared with sham. Tissue from adult naive rats and naive rats at 17 days and 31 days old were also collected. We investigated the expression of transporter gene mRNA at six time points following injury, including 3h, 12h, 24h, 72h, 7d, and 14d. We designed a gene expression panel using the next-generation gene expression methodology from Nanostring.

We found similar magnitudes of expression among brain tissues (hippocampus, cortex, choroid plexus). We also successfully replicated many known expression differences between brain tissues and liver/kidney expression. This study also identified some previously unknown changes - particularly among peptide transporters. Slc15a1 was expressed in the brain at PND-17 and 31, but not in adults. This differs from previously known expression patterns that suggested this transporter is limited to intestinal expression. In addition to the Slc15a2 transporter, Slc15a1 transports small polypeptides and peptide-like drugs (e.g. beta-lactam antibiotics). This might indicate aberration in the disposition of beta-lactam antibiotics post TBI. This study also identified numerous other large and small changes in expression post injury that are of interest in drug development for specific age groups and may help to better understand brain development with different age groups.

Investigation of the expression patterns of transporters post TBI showed that most transporters on our panel showed acute decrease in expression post injury. In com-

parison to sham, over half of the transporters we investigated showed an immediate drop in mRNA expression post TBI. Interestingly, in many cases that was followed with a slow return to baseline or an inflection (i.e. increase in expression from sham). The switch to over-expression was primarily found among the SLC transporters. Many of these change appear to follow changes in transcription factors. IL-6 showed immediate spike in expression with very fast return to baseline in hippocampus and cortex, which is a known regulator of expression of many transporters and other metabolism related genes. Delayed induction of HIF-1a likely explains the slow return to baseline.

This study also identified some interesting expression patterns. The bile acid transporter, Abcb11, is only associated with hepatic expression for obvious reasons. We found that it was induced in the ipsilateral cortex and in the contralateral hippocampus post TBI. It is unclear what role it may play in the brain post injury, though it is possible that it is either 1) not translated to functional protein, or 2) an off-target effect of the inflammatory pathways post injury and has no actual role in the brain. Nevertheless, it is an interesting occurrence.

A final interesting finding was that in all injured tissues, neuroglobin was down-regulated. As mentioned, this is counter to the existing data in this area that suggests that neuroglobin is induced following TBI. It is likely that this finding is connected to the injury model - CCI, and/or the disposition of the rats (e.g. age, time points, etc). Our particular CCI model is fairly void of diffuse injury considering that the skull cap is not replaced and the injury mechanism is very localized. The up-regulation of neuroglobin found in other studies may be tied to diffuse injuries, which are likely to activate other injury pathways.

The final analysis within this study investigated the canonical pathways activated post TBI. Given our relatively small set of genes, highly enriched for transporters, it is

unlikely that we would be able to find many fully defined pathways. However, we did identify enrichment in pathways related to inhibition of brain damage and neuronal cell death. This shows that this injury model causes significant damage as to activate neural repair pathways.

5.1.2 *ABCG2* c.421C>A is Associated with Outcomes Following Severe TBI

For this project, we sought to evaluate the role of the c.421C>A variation in the transporter gene, *ABCG2*, in patients suffering from severe TBI. We sampled nearly 400 patients across two independent cohorts with two years of outcomes collected with the GOS score. Outcomes were compared with genotype controlled for relevant clinical and demographic parameters.

The major finding from this study was that the "CA" genotype at *ABCG2* c.421C>A is associated with improved GOS scores following injury (β=23.56, p<0.001) with a significant age*genotype interaction (β=-6.69, p<0.001). This was replicated in two cohorts and in a single combined cohort. The interaction between genotype and age was an interesting finding. We found that the association of genotype with outcomes was much stronger in individuals, with a diminishing effect as age progress beyond the early 30s. Compared to previous studies of the role of age in TBI recover corroborates this finding, further presenting evidence that age is an important factor in the brain's ability to repair itself from injury.

5.1.3 *ABCG2* c.421C>A is Associated with UA Disposition in CSF in Patients with Severe TBI

Based on our finding that *ABCG2* c.421C>A is associated with outcomes following severe TBI, we sought to investigate the potential mechanistic link in this finding. We hypothesized that the genetic association was related to the ABCG2 substrate, uric acid. Uric acid is an antioxidant that is potentially important in neurologic pathologies. Based on that, we measured uric acid in a subset of the patients in the original association study. This study required the development and validation of an HPLC-UV assay for plasma and CSF. For all measurements, we used the maximum measured uric acid level in the CSF (among several samples per subject) and a single plasma concentration.

The first and primary finding in this study was that the level of uric acid in the CSF was associated with genotype at *ABCG2* c.421C>A. This finding was maintained when controlling for age and sex, and suggested that lower CSF concentrations are predicted by the presence of the "CA" genotype. This finding supports the more recent knowledge that ABCG2 expression on the BCSFB is reverse than that of the BBB (i.e. moves substrates from blood to CSF versus from brain to blood). Therefore, this suggests that decreased function of ABCG2 has a measurable impact of CNS levels of uric acid.

We extended this analysis into the original outcomes measurement. That identified lower CSF concentrations of uric acid as predictive of favorable outcomes following TBI. This is likely in corollary with the original genotype association and makes it difficult to say if this is causative of the better outcomes, but it is certainly an interesting finding.

Finally, we evaluated a surrogate measurement of transport capacity by calculating the ratio of CSF uric acid to plasma uric acid. Theoretically, this ratio would encompass the net effect of passive + active diffusion in and out of the CSF from the blood and the brain parenchyma. Comparing this ratio across genotypes can provide an estimate of how much the flux changes as a result of ABCG2 dysfunction. While not as robust as a transporter flux assay, it provides an *in situ* estimation of the impact of genotype. We found that individuals with the "CA" genotype at *ABCG2* c.421C>A had significantly lower ratios, suggesting decreased CSF concentrations relative to the concentrations in the plasma. Importantly, this indicates an actual change in the disposition of uric acid among CNS barriers and further supports our uric acid hypothesis **Figure 5.1**.

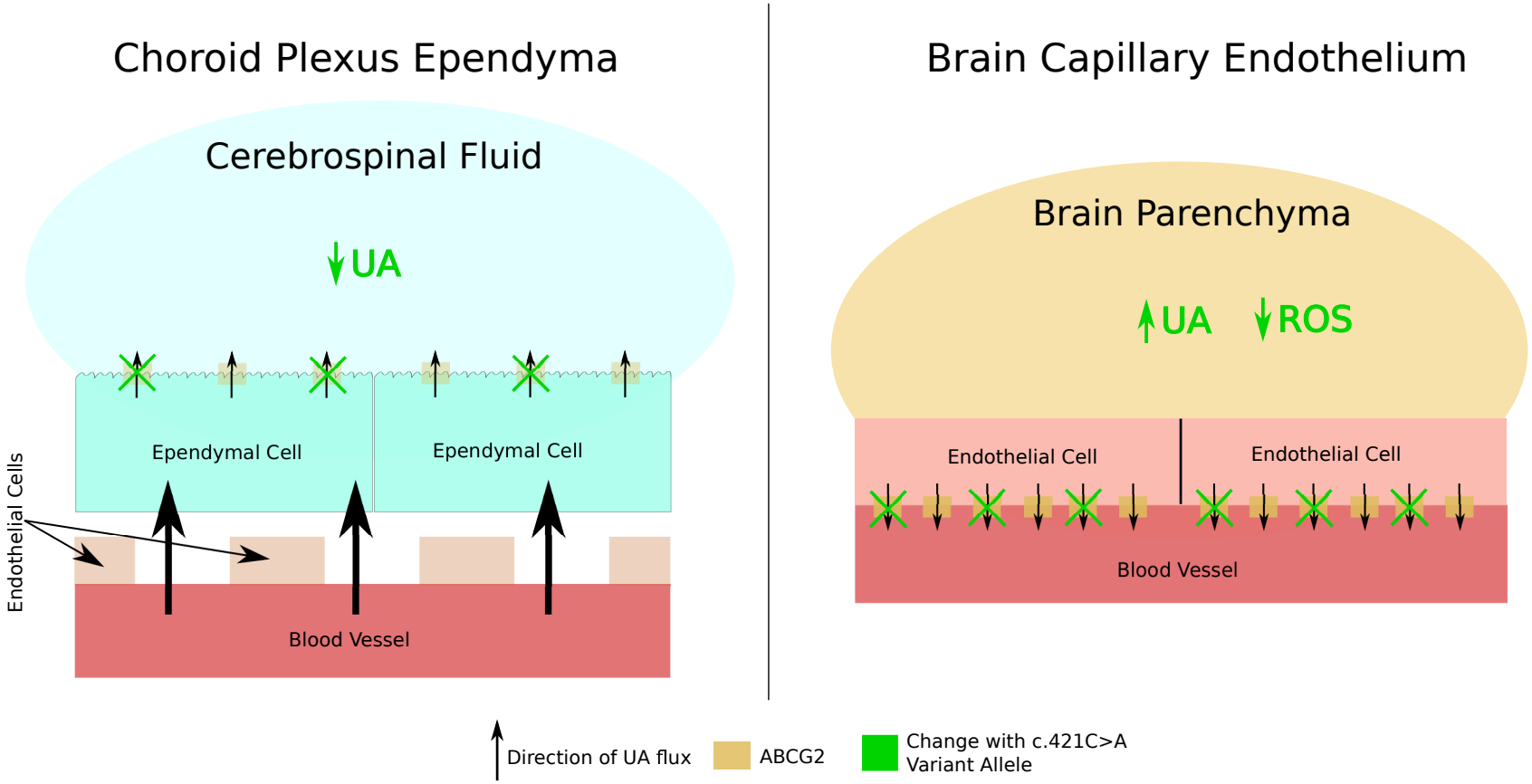

Figure 5.1: Flux of UA with respect to ABCG2 transport in the blood-CSF barrier (left) and BBB (right). At baseline, ABCG2 contributes to the vectoral flux of UA from the blood to the CSF, and from the brain to blood. In the presence of at least one "A" allele at c.421C>A, ABCG2 protein expression is reduced by half, which decreases movement of UA via ABCG2. This predicts lower concentrations relative to blood in the CSF, and higher concentrations relative to blood in the brain. Higher concentrations of UA in the brain suggest decreased oxidative stress due to UA's role as an antioxidant.

5.2 RELEVANCE TO CLINICAL CARE

Traumatic brain injury has no pharmacologic treatments that improve outcomes. Tremendous efforts have been made to research every conceivable molecular pathway associated with secondary injury following TBI with the hope of identifying a successful treatment. Large research groups like Operation Brain Trauma Therapy have developed innovative approaches to better test treatment methodologies.[209] These incorporate different animal models for TBI along with a board of experts to spotlight treatment candidates as a method for rapid translation into clinical trials.

5.2.1 Strategies to Increase Plasma/CSF Uric Acid

An obvious extension of this work is the design of therapeutic interventions that increase uric acid in the CNS. Remarkably, this is not a novel approach as uric acid has been a popular therapeutic target for neuro-pathologies. Methods that have been employed in practice and/or clinical trials include treatment with intravenous uric acid. There is also limited data supporting the use of the purine inosine, which is a uric acid precursor.

Intravenous uric acid has been of interest in the area of neurologic diseases and injuries for several years. Most notably was the urico-ictus trial, a phase 2b-3 clinical trial for uric acid following ischemic stroke.[210] The initial study did not find an association of uric acid therapy with improved outcomes. However, subgroup analysis found that uric acid treatment decreased infarct volume in women.[211] An additional tertiary analysis noted that evidence of benefit of very early treatment with tPA and uric acid in decreasing early ischemic worsening, noting that earlier treatment is more likely to prevent the increase in oxidative stress that occurs after many brain

injuries.[183]

Perhaps a more practical measure of raising uric acid levels for long periods of time is the use of the supplement, inosine. Inosine is a precursor to hypoxanthine and oral supplementation with inosine is able to raise the levels of uric acid in the serum, CSF, and presumably the brain.[212] It is also a naturally occurring compound that is released during times of metabolic stress.[213] Inosine has even been used in a rhesus monkey trial of cortical injury and was found to improve motor function after 14 days of recovery.[213] However, the benefit of inosine in cortical injury is not known to be related to its ability to raise levels of UA. Inosine is an activator of the mammalian Ste20-like protein kinase-3b (Mst3b), which regulates axonal growth.[214, 215] Later work by Lorber and colleagues found that Mst3b is a key regulator of axonal regrowth, suggesting a role in neural plasticity.[216] It is not known if UA, also a purine, has a role in the Mst3b pathway. The protective role of inosine may be related to a combination of Mst3b activation and antioxidant activity of the inosine metabolite, UA.

Inhibition of ABCG2 has been an interest since the discovery that ABCG2 is associated with cancer cell resistance to chemotherapy.[116, 217] The ABCG2 inhibitor, Ko143, is a relatively potent inhibitor for ABCG2; however, its *in vivo* stability has presented challenge in its therapeutic use.[218] Additionally, work by Weidner and colleagues has challenged the specificity of Ko143.[219] They found that, in addition to confirming issues with stability, Ko143 also inhibits the actions of ABCB1 and ABCC1.[219] Successful use of ABCG2 inhibition in TBI would likely require that other transporters, especially ABCB1 and ABCC1, be fully functional. Noteworthy to this point is the association of ABCB1 and ABCC1 decreased function polymorphisms with worse outcomes following TBI.[76] Other lead compounds that are specific ABCG2 inhibitors include PZ-39, fumitremorgin C, and multiple tyrosine kinase inhibitors.[220]

Evaluation of ABCG2 inhibitors capable of producing a pharmacological equivalent of the c.421C>A variation phenotype is warranted.

5.2.2 Age-Related Treatment

A common theme within each part of this study is the relationship of age and recovery from TBI. In the study of gene expression, we focused on a pediatric model for TBI because the recovery from TBI is known to change with age, and changes expression likely differ among different age groups. This is further exemplified in the findings in the final two aims, which showed that the connections of ABCG2 and outcomes are heavily associated with injury. Indeed, the concentrations of uric acid in the serum increase with age, regardless of other clinical parameters.[221] Nearly all studies of the relationship of *ABCG2* c.421C>A have focused on older populations, thus opening the possibility that the contribution of uric acid to recovery following TBI are limited to younger individuals.

5.3 FUTURE RESEARCH DIRECTIONS

5.3.1 Expansion of Expression Study

Animals models for TBI have been an active area for supporting TBI drug development. Our first study of the expression of transporters post injury showed previously unknown patterns of expression changes post injury. Extracting further value from this study could be accomplished by comparing expression changes in human tissue post injury. This can help to better plan drug trials for molecules that may be impacted by the transporters.

5.3.2 Development of Animal Model to study Uric Acid in Experimental TBI

Due to loss of activity of urate oxidase in humans contrasting the preserved activity in most other mammals, rodents are unable to concentrate uric UA in the brain.[195, 194] This makes it challenging to validate our findings in experimental TBI without also accounting for the changes in baseline UA. This would require the development of an experimental TBI model with hyperuricemia. Hyperuricemia can be induced in rodents with supplementation with potassium oxonate, and may be a viable method for measuring this outcome and the impact of uric acid/inosine supplementation in experimental TBI.[222] Urate oxidase knock-out may also be a reasonable way to overcome this limitation, which has been successfully carried out by Lu and colleagues in mice and leads to spontaneous hyperuricemia.[223] This model showed reasonable survival of mice, with nearly 40% still alive at 62 weeks of age.[223]

5.3.3 Validation of Uric Acid CSF Findings

A significant weakness of our study of uric acid concentrations in the CSF and the plasma is the risk for poor sample integrity. The value of bio-banked data and samples cannot be overstated, but findings in decades old samples ought to receive validation from more robust methods. Our study also relied on the use of CSF collected from drainage bags, which is frequently contaminated with blood and raises questions about the room temperature stability of samples. Overall, our study was conducted pragmatically, but would benefit from more robust follow-up. This could also aim to correct the selection bias that might have influenced our results by collecting samples from the entire cohort used in chapter three. Additionally, a study of prospectively collected CSF samples from patients with TBI along with prospective outcomes collection

would be of tremendous value in validating these findings.

5.3.4 Clinical Trial of Uric Acid Raising Therapy

Nevertheless, this study uncovered an important contribution from ABCG2 that is likely related to the benefits of having higher concentrations of uric acid in the brain. Given the accessibility and safety of inosine and uric acid therapy, a clinical trial in patients with severe TBI may be appropriate. Alternatively, these may be given in the previously mentioned rat model for hyperuricemia with experimental brain injury. However, it is likely that hyperuricemic animal models will not have improved outcomes as it is likely that other changes are necessary for an organism to actually benefit from having higher baseline uric acid levels. Therefore, a human study is both ethically and scientifically reasonable.

5.4 COMMENTARY AND CONCLUSION

In chapter two we discussed the differences in baseline expression in of ABCG2 in the brain in humans versus other mammals. Despite having a similar role in these animals, humans tend to have much lower brain expression of ABCG2. Perhaps this is related to a the much higher concentration of UA in human plasma and presumably, other tissues. It suggests that human physiology regarding ABCG2 is tightly related to maintaining high levels of uric acid without going too high. This argument is further supported by the reality that animal models of hyperuricemia are associated with inherent health problems. We propose that differential expression of ABCG2 in human tissues is tied to maintenance of particular UA levels in tissues, particularly the brain.

Genetic variations exist as a natural function of genetics. As per the tenets of evolution, somatic mutations and germline variations do not necessarily exist for a reason beyond chance, but their tendency to persist within a given population can point to either 1) benign phenotype or 2) survival advantage. *ABCG2* c.421C>A is relatively common within the population (MAF = 0.1036) and despite its increase in the risk for gout, does not have any pathogenic association that is known.[136] The variant is also present in virtually every population, suggesting that, despite its functional status, is not faced with negative selection pressure. While this alone does not suggest a survival advantage, it does raise interesting question of whether this variation supports an enhanced neuro-protective role of UA. Deeper analysis of the potential evolutionary role of ABCG2 and this variation are beyond the scope of this dissertation, but do pose interesting questions for better understanding human brain development.

Overall, we sought to determine the connection of transporters and TBI, with a focus on the role of ABCG2 post injury. Transporters have an important role in normal and pathogenic brain physiology, and ABCG2 is central to many of those effects. The connection of ABCG2 to injury progression and recovery identify uric acid as a player following TBI. It supports antioxidant reserve as a viable treatment target for TBI, which has already been a popular target with trials existing targeting glutathione with N-Acetylcysteine. This overall research project has extended the knowledge of the contribution of transporters to recovery following brain injuries, and has provided new research outlets into the role of uric acid in the brain.

APPENDIX A

EXTENDED NANOSTRING DATA

Table A1: Ipsilateral Hippocampus Normalization Comparison

CodeCount.method	Background.method	SampleContent.method	cv.pos.results	cv.hk.results	cv.end.results	cv.bio2tech.ratio
geo.mean	max	housekeeping.geo.mean	1.7	1.3	15.3	9
geo.mean	max	housekeeping.sum	1.6	1.4	15.1	9.44
geo.mean	max	low.cv.geo.mean	1.6	1.7	15.1	9.44
geo.mean	max	top.geo.mean	2.9	3.6	15.6	5.38
geo.mean	max	top.mean	7.8	8.7	20.8	2.67
geo.mean	max	total.sum	7.8	8.7	20.8	2.67
geo.mean	mean	housekeeping.geo.mean	1.7	1.2	14.3	8.41
geo.mean	mean	housekeeping.sum	1.6	1.4	14.3	8.94
geo.mean	mean	low.cv.geo.mean	1.5	1.5	14.1	9.4
geo.mean	mean	top.geo.mean	2.3	3.1	13.5	5.87
geo.mean	mean	top.mean	7.7	8.6	18.7	2.43
geo.mean	mean	total.sum	7.7	8.6	18.7	2.43
geo.mean	mean.2sd	housekeeping.geo.mean	1.7	1.3	15.5	9.12
geo.mean	mean.2sd	housekeeping.sum	1.7	1.5	15.2	8.94
geo.mean	mean.2sd	low.cv.geo.mean	1.6	1.7	15.3	9.56
geo.mean	mean.2sd	top.geo.mean	2.9	3.7	15	5.17
geo.mean	mean.2sd	top.mean	7.8	8.7	19.7	2.53
geo.mean	mean.2sd	total.sum	7.8	8.7	19.7	2.53

Table A2: Ipsilateral Cortex Normalization Comparison

CodeCount.method	Background.method	SampleContent.method	cv.pos.results	cv.hk.results	cv.end.results	cv.bio2tech.ratio
geo.mean	max	housekeeping.geo.mean	3.1	0.9	15.3	4.94
geo.mean	max	housekeeping.sum	3.1	1	15.4	4.97
geo.mean	max	low.cv.geo.mean	3.2	1.2	15.1	4.72
geo.mean	max	top.geo.mean	3.3	2.5	14.3	4.33
geo.mean	max	top.mean	7.8	6.9	16.7	2.14
geo.mean	max	total.sum	7.8	6.9	16.7	2.14
geo.mean	mean	housekeeping.geo.mean	3.1	0.9	19.4	6.26
geo.mean	mean	housekeeping.sum	3.1	1	19.3	6.23
geo.mean	mean	low.cv.geo.mean	3.2	1.2	19.2	6
geo.mean	mean	top.geo.mean	3.3	2.5	18.6	5.64
geo.mean	mean	top.mean	7.7	6.8	21.2	2.75
geo.mean	mean	total.sum	7.7	6.8	21.2	2.75
geo.mean	mean.2sd	housekeeping.geo.mean	3.1	0.9	13.6	4.39
geo.mean	mean.2sd	housekeeping.sum	3.1	1	14	4.52
geo.mean	mean.2sd	low.cv.geo.mean	3.2	1.2	13.8	4.31
geo.mean	mean.2sd	top.geo.mean	3.3	2.5	13.3	4.03
geo.mean	mean.2sd	top.mean	7.8	6.9	16.4	2.1
geo.mean	mean.2sd	total.sum	7.8	6.9	16.4	2.1

Table A3: Raw Naïve Count Data (Part 1)

Age	ID	Tissue	Abcb11	Abcb1a	Abcb1b	Abcc1	Abcc2	Abcc4	Abcc5	Abcc8	Abcg2	Slc15a1	Slc15a2	Slc16a1	Slc22a2
17	EMPXP-17	Choroid Plexus	0	9.888	3.253	8.541	6.172	8.09	8.155	6.946	8.393	0	9.291	11.63	7.39
17	EMPXP-41	Choroid Plexus	0	10.29	1.066	8.987	4.005	8.679	8.178	5.631	7.585	0	8.837	11.72	9.546
17	EMPXP-57	Choroid Plexus	0	8.166	3.765	9.983	4.823	8.324	7.557	5.998	5.277	5.294	8.324	10.52	6.94
17	EMPXP-89	Choroid Plexus	0	9.572	0	8.79	0	7.22	7.837	5.842	6.556	0	8.905	11.14	8.708
31	EMPXP-33	Choroid Plexus	0	6.405	3.516	9.354	4.173	7.73	6.771	5.747	4.928	5.285	7.308	8.925	5.51
31	EMPXP-42	Choroid Plexus	0	9.632	1.852	9.765	4.717	8.151	7.728	5.111	6.921	5.89	7.924	9.466	9.352
31	EMPXP-65	Choroid Plexus	0	9.244	0	8.481	4.223	7.305	8.264	6.293	6.886	0	8.597	10.8	10.55
31	EMPXP-81	Choroid Plexus	0	7.304	2.103	10.07	4.366	8.532	6.93	3.588	5.272	5.889	7.836	8.366	7.626
17	EMPXP-17	Cortex	0	8.744	4.73	9.327	7.678	7.415	9.835	8.796	8.071	3.599	9.045	10.86	4.321
17	EMPXP-41	Cortex	0	8.165	4.755	8.833	7.197	6.91	9.343	8.337	6.766	2.531	8.948	10.57	4.439
17	EMPXP-57	Cortex	0	8.734	5.281	9.067	7.403	7.348	9.636	8.576	6.508	2.869	8.867	11.39	4.259
17	EMPXP-89	Cortex	0	8.687	4.544	8.834	7.17	7.149	9.399	8.509	7.485	1.577	8.721	10.61	4.408
31	EMPXP-33	Cortex	0	8.753	4.31	9.033	7.709	6.912	9.756	8.572	6.727	0.7653	8.789	10.28	4.436
31	EMPXP-42	Cortex	0	9.073	3.588	9.254	7.875	6.54	9.754	8.418	7.083	0	9.244	9.87	4.966
31	EMPXP-65	Cortex	0	9.035	4.6	8.932	7.744	7.051	9.556	8.292	6.593	0	9.029	10.19	5.077
31	EMPXP-81	Cortex	0	8.864	3.754	9.148	7.984	6.914	9.752	8.708	6.549	0	8.873	10.46	5.126
A	EMPXP-100	Cortex	0	9.443	3.634	8.961	8.119	6.519	9.68	8.424	7.773	0	9.351	9.865	4.215
A	EMPXP-101	Cortex	0	9.431	4.469	9.356	8.065	7.23	9.884	8.57	6.781	0	9.27	10.03	4.812
A	EMPXP-102	Cortex	0	9.198	3.339	9.109	7.818	6.262	9.793	8.323	7.714	0	8.994	9.933	4.725
17	EMPXP-17	Hippocampus	0	7.964	1.865	9.477	7.17	7.445	10.17	8.205	7.813	4.144	8.89	11.06	3.74
17	EMPXP-41	Hippocampus	0	8.208	8.557	9.576	6.968	7.705	10.47	8.801	7.209	5.343	9.59	10.78	3.36
17	EMPXP-57	Hippocampus	0	8.506	8.157	9.344	7.321	7.347	10.17	8.645	7.082	4.875	9.357	11.19	5.324
17	EMPXP-89	Hippocampus	0	8.561	8.174	9.213	7.299	7.203	10.43	8.557	7.841	4.425	9.463	10.71	4.087
31	EMPXP-33	Hippocampus	0	8.326	8.4	9.49	7.129	7.045	10.56	8.827	6.362	4.972	9.634	10.42	4.481
31	EMPXP-42	Hippocampus	0	8.263	8.523	9.303	7.22	6.865	10.57	8.412	6.949	4.842	9.828	9.974	5.008
31	EMPXP-65	Hippocampus	0	8.389	7.807	9.219	7.794	7.29	10.47	8.633	6.677	4.875	9.757	10.52	3.411
31	EMPXP-81	Hippocampus	0	8.42	7.583	9.055	7.654	7.054	10.57	8.425	6.576	4.749	9.762	10.6	5.232
A	EMPXP-100	Hippocampus	0	9.328	8.798	9.308	7.801	6.736	9.838	8.852	8.001	0	9.856	10.17	4.713
A	EMPXP-101	Hippocampus	0	9.142	8.628	9.393	7.964	6.966	9.902	8.707	6.627	0	9.856	10.13	4.977
A	EMPXP-102	Hippocampus	0	9.416	8.119	9.237	8.202	6.496	10.04	8.974	8.114	2.855	9.64	10.27	4.749
A	EMPXP-103	Hippocampus	0	9.417	8.295	9.352	8.022	6.98	10.09	9.059	7.157	1.511	9.525	10.32	4.332
17	EMPXP-17	Kidney	0	7.136	3.21	9.073	7.237	9.501	9.358	0	9.165	5.532	9.632	8.572	9.718
17	EMPXP-41	Kidney	8.86	6.856	0	8.979	9.154	9.31	8.875	0	8.826	5.026	9.356	9.712	9.51
17	EMPXP-57	Kidney	0	7.011	2.542	9.063	7.294	9.283	8.41	0	8.885	5.245	9.865	8.692	9.824
17	EMPXP-89	Kidney	2.987	7.144	2.316	9.046	7.496	9.224	9.262	0	9.252	5.412	10.16	8.396	9.836
31	EMPXP-33	Kidney	4.312	7.7	2.256	8.824	7.925	9.989	7.734	0	10.94	5.909	10.39	9.231	12.94
31	EMPXP-42	Kidney	3.843	7.894	2.856	8.791	7.856	10.09	7.224	0	10.78	5.641	10.67	7.918	12.89
31	EMPXP-65	Kidney	2.36	7.998	3.36	8.717	7.914	9.769	7.638	0	10.73	5.896	10.41	8.203	12.76
31	EMPXP-81	Kidney	3.58	7.605	0	8.707	7.718	9.52	7.501	0	10.79	4.802	10.27	9.753	12.52
17	EMPXP-17	Liver	11.19	7.143	0.224	6.201	11.17	4.031	9.041	0	8.918	3.224	1.809	11.1	1.809
17	EMPXP-41	Liver	11.2	6.854	0	5.739	10.61	3.656	8.985	0	9.161	4.099	0	11.1	3.013
17	EMPXP-57	Liver	10.88	7.537	1.952	6.06	10.84	4.2	8.985	0	8.859	3.122	0	11.26	0
17	EMPXP-89	Liver	10.62	6.77	0	5.786	10.43	4.064	8.851	0	8.421	2.673	0	10.51	0
31	EMPXP-33	Liver	12.48	8.737	6.369	5.745	12.64	6.619	5.067	0	9.274	4.745	0	11.59	0
31	EMPXP-42	Liver	11.83	8.944	5.685	5.482	12.69	7.841	5.482	0	8.825	4.002	0	10.5	1.754
31	EMPXP-65	Liver	11.95	8.158	5.34	5.279	12.45	6.306	5.535	0	8.618	4.461	0	10.77	0
31	EMPXP-81	Liver	11.93	8.41	7.214	4.991	12.21	6.322	5.034	0	9.159	4.532	2.947	11.17	0

Table A4: Raw Naïve Count Data (Part 2)

Age	ID	Tissue	Slc22a4	Slc22a6	Slc22a8	Slc28a2	Slc29a1	Slc2a1	Slc47a1	Slc7a1	Slc7a5	Slco1a1	Slco1a2	Slco1a5	Slco1b2	Slco2b1
17	EMPXP-17	Choroid Plexus	7.157	10.02	9.588	6.568	8.028	12.17	7.547	7.797	9.744	0	9.372	2.316	0	9.058
17	EMPXP-41	Choroid Plexus	7.564	11.71	10.28	7.358	8.718	12.38	8.136	7.626	9.66	0	10.89	1.066	0	9.124
17	EMPXP-57	Choroid Plexus	6.374	8.866	11.39	5.828	8.105	10.54	6.794	6.711	8.534	0	7.899	12.57	0	7.273
17	EMPXP-89	Choroid Plexus	7.097	10.37	9.046	6.354	8.849	11.95	8.505	5.842	9.864	0	8.924	0	0	7.798
31	EMPXP-33	Choroid Plexus	5.758	7.687	10.94	6.161	7.127	10.1	8.321	4.752	8.437	0	6.845	12.28	2.763	5.969
31	EMPXP-42	Choroid Plexus	6.078	10.94	11.82	7.189	7.742	11.14	10.03	5.839	8.14	0	10.51	12.23	0	8.582
31	EMPXP-65	Choroid Plexus	8.064	11.96	11.12	8.31	8.597	11.08	9.773	4.223	8.597	0	11.1	0	0	8.738
31	EMPXP-81	Choroid Plexus	6.508	9.192	11.76	5.207	7.19	10.08	7.752	4.366	8.177	1.781	8.741	13.05	0	6.847
17	EMPXP-17	Cortex	4.73	7.175	8.957	8.441	7.108	10.92	4.118	8.753	9.558	0	7.3	3.882	2.09	8.374
17	EMPXP-41	Cortex	4.162	6.928	8.527	7.604	6.607	10.36	4.489	8.475	9.364	0	7.219	0	2.709	8.285
17	EMPXP-57	Cortex	5.159	6.852	8.971	7.927	6.947	10.43	5.394	9.234	9.355	0	7.721	0.8293	2.704	8.471
17	EMPXP-89	Cortex	4.257	6.777	8.738	8.086	6.701	10.81	4.669	8.537	9.419	0	7.03	0	2.699	7.785
31	EMPXP-33	Cortex	5.378	7.124	8.891	8.378	6.471	10.49	5.346	8.053	9	0	8.458	0	1.813	7.971
31	EMPXP-42	Cortex	4.966	7.321	8.986	8.136	7.094	10.63	5.371	7.799	9.162	0	8.665	0	0.9249	8.384
31	EMPXP-65	Cortex	5.916	6.593	8.441	7.732	6.779	10.51	3.682	8.21	8.92	0	8.72	0	2.834	8.433
31	EMPXP-81	Cortex	5.339	6.806	8.801	8.232	6.952	10.61	5.448	8.524	9.044	0	8.866	0	1.988	8.377
A	EMPXP-100	Cortex	5.533	6.352	8.662	7.913	6.862	10.66	3.443	7.788	8.818	0	8.708	2.645	1.267	8.216
A	EMPXP-101	Cortex	6.298	6.365	8.948	8.474	7.064	10.72	1.481	8.096	9.006	0	8.948	7.38	3.355	8.266
A	EMPXP-102	Cortex	6.274	5.885	8.387	8.045	6.647	10.57	4.451	8.016	8.679	0	8.579	0	2.086	8.135
17	EMPXP-17	Hippocampus	5.446	4.31	8.697	5.765	8.628	11.07	0	8.055	9.619	0.5698	7.848	7.757	0	8.551
17	EMPXP-41	Hippocampus	5.627	6.247	8.498	7.457	5.985	10.9	1.425	8.877	9.972	0	7.953	4.051	0	8.533
17	EMPXP-57	Hippocampus	4.875	7.051	8.951	7.355	6.286	10.62	5.215	9.155	9.842	1.049	8.172	4.43	0	8.574
17	EMPXP-89	Hippocampus	4.348	5.872	8.454	7.278	5.757	11.26	0.3379	8.353	9.652	1.812	8.008	4.499	0	8.477
31	EMPXP-33	Hippocampus	6.458	5.377	7.957	7.324	5.628	10.96	4.06	8.461	9.508	0	8.977	4.406	0	8.627
31	EMPXP-42	Hippocampus	6.073	5.531	8.011	7.592	5.261	10.81	1.672	8.05	9.366	1.672	9.07	3.472	0	8.544
31	EMPXP-65	Hippocampus	7.016	6.353	8.148	7.585	6.081	10.92	5.558	8.254	9.774	0	9.089	4.103	0	8.853
31	EMPXP-81	Hippocampus	6.348	6.855	8.537	7.55	6.145	10.89	5.104	8.197	9.202	1.185	9.293	6.576	0	8.393
A	EMPXP-100	Hippocampus	6.708	3.826	8.035	7.99	6.84	10.88	1.093	8.7	9.351	0	9.043	2.05	0.1759	8.788
A	EMPXP-101	Hippocampus	7.179	3.176	7.898	8.256	6.994	10.78	0	8.325	9.311	0	8.934	0	1.488	8.632
A	EMPXP-102	Hippocampus	7.189	5.541	8.26	8.392	7.1	10.81	5.435	8.531	9.187	0	8.766	0	2.336	8.526
A	EMPXP-103	Hippocampus	7.007	4.387	7.855	8.478	7.109	10.92	1.511	8.594	9.344	0	9.016	0	2.761	8.423
17	EMPXP-17	Kidney	7.789	12.09	12.96	5.532	7.007	9.477	11.01	6.877	5.911	2.474	2.889	4.412	2.696	7.905
17	EMPXP-41	Kidney	7.359	11.83	12.76	7.166	7.117	9.211	10.69	5.826	5.944	7.04	7.178	4.731	9.984	8.986
17	EMPXP-57	Kidney	7.677	11.99	12.86	5.284	6.916	9.283	11.09	6.129	5.593	0	3.158	4.105	2.542	7.413
17	EMPXP-89	Kidney	7.826	11.66	12.77	6.215	6.951	9.824	11.28	6.403	5.823	0.368	4.131	3.789	4.455	8.563
31	EMPXP-33	Kidney	8.742	12.77	12.85	5.834	6.09	9.444	12.27	4.997	5.321	10.89	1.915	4.383	2.763	6.705
31	EMPXP-42	Kidney	8.678	12.58	12.79	5.641	5.581	9.292	12.14	4.836	5.699	10.78	3.362	4.121	2.637	6.31
31	EMPXP-65	Kidney	8.638	12.71	12.94	5.497	6.247	9.424	12.21	5.845	5.592	10.56	3.208	3.738	2.845	6.696
31	EMPXP-81	Kidney	8.359	12.45	12.53	5.494	6.376	9.259	12.04	4.802	5.089	10.34	3.72	1.102	3.049	6.266
17	EMPXP-17	Liver	3.924	0	10.46	8.595	5.509	6.853	0	0	6.414	9.091	8.999	5.031	12.56	10.65
17	EMPXP-41	Liver	4.511	0	10.24	8.828	3.202	6.989	0	0	6.411	9.217	8.929	3.894	12.23	10.58
17	EMPXP-57	Liver	3.759	0	10.09	8.102	5.344	6.652	0	0	6.581	9.38	9.658	4.537	12.4	10.52
17	EMPXP-89	Liver	4.186	0	9.372	8.943	4.299	6.354	0	0	6.808	9.187	8.79	2.973	12.06	10.19
31	EMPXP-33	Liver	6.888	0	12.72	10.17	7.832	6.289	0	0	5.967	11.01	11.9	5.247	14.1	10.91
31	EMPXP-42	Liver	6.369	0	11.98	9.058	6.983	6.076	0	0.754	6.398	10.43	11.28	4.076	13.27	10.33
31	EMPXP-65	Liver	6.423	0	12.22	9.413	8.079	6.178	0	0	5.84	10.52	11.06	4.461	13.66	10.63
31	EMPXP-81	Liver	6.754	1.947	11.93	9.777	7.423	5.754	0	1.532	7.147	10.53	11.22	5.076	13.2	10.49

APPENDIX B

EXTENDED DATA FROM ABCG2 GENETIC ASSOCIATION

```
EstimatePropOdds <- function(model_list) {
  require(reshape)
  LL.df <- data.frame()
  UL.df <- data.frame()
  params <- c()
  i <- 1
  for (model in model_list) {
    se <- sqrt(diag(vcov(model)))
    LL <- data.frame(t(fixef(model) - 1.96 * se))
    UL <- data.frame(t(fixef(model) + 1.96 * se))
    params <- names(LL)
    LL$model <- i
    UL$model <- i
    LL.df <- rbind(LL.df, LL)
    UL.df <- rbind(UL.df, UL)
    i <- i + 1
  }
  LL.df$limit <- "lower"
  UL.df$limit <- "upper"
  limits <- rbind(LL.df, UL.df)
  for (param in params) {
    rows <- reshape(
      data = limits[, c(param, "limit", "model")],
      direction = "wide",
      timevar = "limit",
      idvar = "model"
    )
```

```
    intervals <-
      matrix(
        data = c(rows[, paste(param, "upper", sep = ".")],
                 rows[, paste(param, "lower", sep = ".")]),
        ncol = 2,
        nrow = length(model_list)
      )
    # Recursively test for interval overlap

    apply(intervals, 1, function(v0) {
      apply(intervals, 1, function(v1) {

        if (v0[1] < v1[2] & v0[2] < v1[2]){
          pass <- FALSE
        }
        else if (v0[1] > v1[1] & v0[2] > v1[1]) {
          pass <- FALSE
        }
        else {
          pass <- TRUE
        }
        if (!pass) {
          print(paste(param, "Failed!", sep = " - "))
        }
      })
    })
  }
  return(rbind(LL.df, UL.df))
}
prop1_2345 <- glmer(data = proptest,
                    GOS1_2345 ~ rs2231142_dc * log(Age) +
                      Sex + Time + None + EDH + SDH + SAH +
                      IPH + IVH + DAI + no_data + GCS + (1|IDENT),
                    nAGQ = 0, family = binomial, control = glmerControl(optimizer
                       = "bobyqa", optCtrl = list(maxfun=2e5)),
                    verbose = TRUE)

prop12_345 <- glmer(data = proptest,
                    GOS12_345 ~ rs2231142_dc * log(Age) +
                      Sex + Time + None + EDH + SDH + SAH +
                      IPH + IVH + DAI + no_data + GCS + (1|IDENT),
```

```
                nAGQ = 0, family = binomial, control = glmerControl(optimizer
                   = "bobyqa", optCtrl = list(maxfun=2e5)),
                verbose = TRUE)

prop123_45 <- glmer(data = proptest,
                GOS123_45 ~ rs2231142_dc * log(Age) +
                  Sex + Time + None + EDH + SDH + SAH +
                  IPH + IVH + DAI + no_data + GCS + (1|IDENT),
                nAGQ=0, family = binomial, control = glmerControl(optimizer =
                   "bobyqa", optCtrl = list(maxfun=2e5)),
                verbose = TRUE)

prop1234_5 <- glmer(data = proptest,
                GOS1234_5 ~ rs2231142_dc * log(Age) +
                  Sex + Time + None + EDH + SDH + SAH +
                  IPH + IVH + DAI + no_data + GCS + (1|IDENT),
                nAGQ = 0, family = binomial, control = glmerControl(optimizer
                   = "bobyqa", optCtrl = list(maxfun=2e5)),
                verbose = TRUE)
ci_table <- EstimatePropOdds(c(prop1_2345, prop12_345, prop123_45,
   prop1234_5))
```

APPENDIX C

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Figure C1: Future Medicine Pharmacogenomics Release

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153

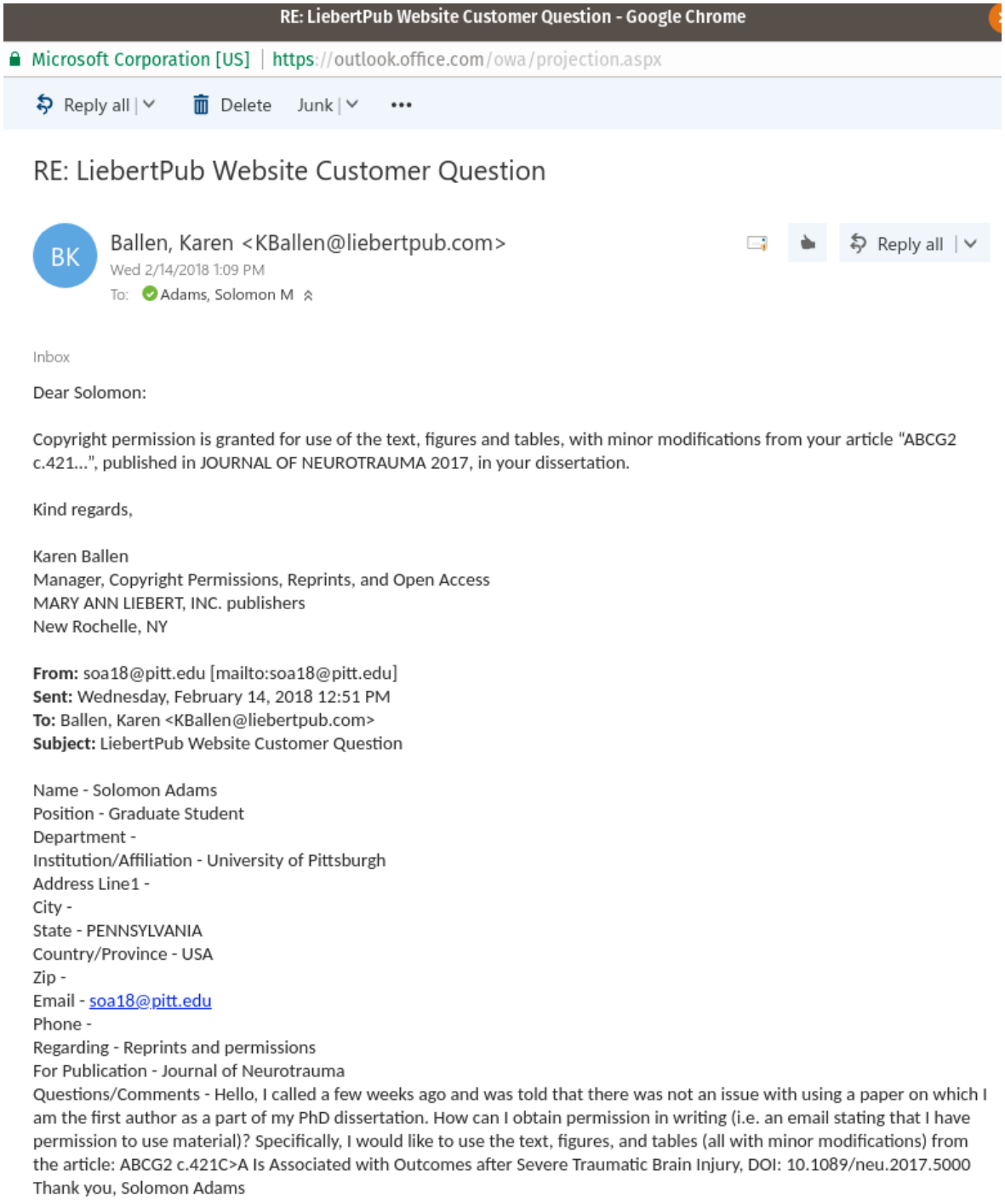

Figure C2: Journal of Neurotrauma Release

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